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PRODUCTION OF VOLATILE SULFUR COMPOUNDS FROM INORGANIC
SULFUR BY LACTOCOCCI

by

Supriyo Ghosh

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

2003

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ABSTRACT

Production of Volatile Sulfur Compounds from Inorganic

Sulfur by Lactococci

by

Supriyo Ghosh, Master of Science

Utah State University, 2003

Major Professor: Dr Bart C. Weimer
Department: Nutrition and Food Sciences

Production of volatile sulfur compounds in cheese is associated with desirable flavors. The direct source of these compounds has been assumed to arise from the metabolism of methionine and cysteine. However, the methionine concentration in cheese rises above the amount found in casein during aging, suggesting that alternative sulfur sources are present in milk. This led us to hypothesize that lactococci may acquire sulfur from the inorganic sulfur pool of milk, in addition to methionine and cysteine, to generate volatile sulfur compounds during cheese ripening.

A turbidimetric method to determine total sulfate content in milk samples was developed. The average sulfate content of milk was determined to be $\sim 49 \text{ mg/L} \pm 2.0 \text{ mg/L}$. The limit of detection of the test was $\sim 2.5 \text{ mg/L}$ in Tris buffer and $\sim 10 \text{ mg/L}$ in milk. Skim milk samples had significantly higher total sulfate content as compared to whole milk samples.

Transport of sulfate by three strains of *Lactococcus* sp. was studied after we determined that milk had small, but measurable amounts of inorganic sulfate. A decrease in the environmental pH increased sulfate transport. The maximum transport occurred during exponential cellular growth phase. All strains tested had the ability to transport much more sulfate than is native in milk.

The last phase of study was to determine the metabolic fate of sulfate. Incorporation of radio-labeled sulfate into cellular protein was studied by two-dimensional gel-electrophoresis of crude cellular lysate followed by auto-radiography. Production of volatile sulfur compounds from inorganic sulfur was determined with analysis of the head space gas with gas chromatography and scintillation counting. The incorporation of radio-labeled sulfur from sulfate was not detected in proteins on two-dimensional gels. Detectable volatile sulfur compounds were found only in the case of gas chromatographic analysis of ML3 head space. However, radio-labeled volatile sulfur was detected in the head space of all the three strains with scintillation counting. This study defined that lactococci can fix inorganic sulfur into volatile sulfur compounds in small amounts.

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LIST OF SYMBOLS, NOTATIONS, DEFINITIONS

Abbreviation key

2-D gel = two-dimensional gel electrophoresis

CDM = chemically defined medium

Cys = cysteine

DMDS = dimethyl disulfide

DMS = dimethyl sulfide

DMTS = dimethyl trisulfide

GC = gas chromatography

IEF = iso-electric focusing

KMTB = α - keto γ - thiomethyl butyrate

Met = methionine

MGL = L- methionine γ - lyase

MTB = methyl thiobutyrate

OD = optical density

RSD = relative standard deviation

SAM = S-adenosyl methionine

SDS = sodium dodecyl sulfate

VSC = volatile sulfur compound

CHAPTER I

INTRODUCTION

A particular balance of specific products from microbial and chemical reactions in cheese, lead to its typical flavor. Sulfur compounds, particularly, comprise a distinct fraction of Cheddar cheese flavor and aroma that is associated with desirable flavors (8, 9). Grill et al. (12), using reagents to trap specific compounds in Cheddar cheese slurries, determined that elimination of sulfur compounds adversely affects the aroma of Cheddar cheese. Methanethiol, specifically, positively correlates to flavorful Cheddar cheese.

Cysteine and methionine are the sulfur-containing amino acids in milk that are converted to volatile sulfur compounds (VSC) during cheese ripening. Cysteine has four residues in all caseins and methionine has 16 residues that remain in the cheese matrix (5). This is a very small amount of starting material for production of VSCs to contribute to cheese flavor. Wallace and Fox (24) added casein-amino acids to cheese during manufacture in an effort to improve volatile sulfur compound production during cheese ripening and hence flavor enhancement. In all the cheese samples, after ripening, the free methionine content was approximately 10-fold above the control. This increase is much higher than that can be accounted for with the number of residues in the casein molecules. The source of the additional sulfur atoms in the volatile fraction is unknown. This led us to hypothesize that inorganic sulfur is a potential source of the sulfur atom.

Analysis of the total genome sequence of *Lactococcus lactis* IL1403 demonstrated this organism has several genes for sulfate assimilation, but not all of them those are needed in the entire fixation pathway. These sulfate fixation pathways are well known in

E. coli and *Pseudomonas aeruginosa*. This analysis further suggested that lactococci have to the potential to assimilate inorganic sulfate via an undefined mechanism.

To verify this hypothesis, we began by developing a turbidimetric method to determine total sulfate content of milk. Subsequently, the influence of environmental conditions and cellular growth phase were investigated for sulfate transport by lactococcal strains. The assimilation of sulfate into cellular protein was assessed, but was not observed. The profile of total VSC produced by lactococcal strains in assays with different levels of sulfate was quantified in the headspace of assays containing sulfate, methionine, and cysteine. Lastly, the VSC compounds from fixed sulfur, was observed with scintillation counting, but not with gas chromatography. This work determined that lactococci fixed inorganic sulfur at low levels, thereby proving the hypothesis of the study.

LITERATURE REVIEW

Cheese Flavor

Flavor development in cheese is promoted by the microbial catabolism of amino acids, resulting from primary proteolysis (23). The peptides from the cheese curd are transported into the cell and hydrolyzed by aminopeptidases to free amino acids, destined to catabolic products. A particular balance among the specific products of microbial and chemical reactions in the cheese with specific concentrations, lead to the typical flavor of Cheddar cheese. Sulfur compounds including hydrogen sulfide, dimethyl disulfide, methanethiol, and methional comprise a distinct fraction of Cheddar cheese flavor and aroma (Table 1).

Grill et al. (12), using reagents to trap specific compounds in Cheddar cheese slurries, found that elimination of sulfur compounds adversely affected the aroma of Cheddar cheese. Methanethiol in particular correlates to flavorful cheddar cheese. Gas chromatographic analysis in conjunction with sensory evaluation of Cheddar cheese distillates maintained at low temperature also showed that sulfur compounds significantly contribute to the aroma of Cheddar cheese as well (19). These volatile sulfur compounds have very high odor activity values (13, 18, 21); hence minuscule amounts of these compounds have a profound influence on cheese flavor and aroma.

TABLE 1. Detection thresholds and properties of sulfur compounds found in cheese.

Compound	Detection Threshold (ppb)	Sensory Medium	Flavor/aroma Description	Probable Precursor	Reference
Hydrogen sulfide	0.18	air	rotten eggs	cysteine	5
Carbonyl sulfide				cysteine	5
Methanethiol	2	water	cooked cabbage	methionine	19
Methional	21	Skim milk	boiled potato	methionine	14
Dimethyl sulfide	20	milk	cabbage		19
Dimethyl disulfide	20	Skim milk	cauliflower,	methanethiol	14
	1.2	water	garlic, very ripe cheese		
Dimethyl trisulfide	0.01	water		methanethiol, formaldehyde	23
S-methyl thioacetate	5	liquid cheese	cooking cauliflower	methanethiol, acetic acid	4
S-methyl thiopropionate	100	liquid cheese	cheesy	methanethiol, propionic acid	4
S-methyl thiobutyrate	100	liquid cheese	chives	methanethiol, butyric acid	4

Methanethiol (CH₃-SH)

Methanethiol is a volatile (boiling point of 6.2°C) compound possessing an intense putrid, fecal-like aroma, at high concentrations. It is implicated as an influential aroma and flavor compound in many foods, including some surface-ripened cheeses like

Limburger, and Muenster. Several researchers have used convincing evidence to implicate methanethiol in good Cheddar cheese flavor (19). Cheese made aseptically with starter cultures develop normal Cheddar flavor and methanethiol is detected in their distillates (19). However, cheeses made by direct acidification with α -gluconic acid δ -lactone in the absence of starter cultures do not contain methanethiol in the headspace and lack typical Cheddar-type flavor. Furthermore, removal of thiols, including

methanethiol, from a nitrogen stream passed through a Cheddar slurry results in the destruction of Cheddar aroma, indicating that thiols play an important role in Cheddar cheese aroma. However, methanethiol is not “the” Cheddar cheese flavor compound, as its presence alone does not cause true Cheddar-like flavor notes in experimental cheese.

Dimethyldisulfide ($\text{CH}_3\text{-S-S-CH}_3$)

Methanethiol readily oxidizes to dimethyldisulfide and dimethyltrisulfide. Additionally, direct production of dimethyldisulfide from methionine via an unknown carrier compound may also be possible. The role of this compound in cheese flavor is ambiguous, with some authors claiming it positively influences cheese flavor and while others questioning its significance (23).

Dimethyltrisulfide ($\text{CH}_3\text{-S-S-S-CH}_3$)

Dimethyltrisulfide is not associated with a specific flavor, but it is commonly found in the headspace of cheese (20). Occurrence of this compound is a direct result of methanethiol content and is modulated by the low redox potential present in cheese. Dias and Weimer (5) found methanethiol to decrease as this compound increased in the headspace of Cheddar cheese slurries.

Dimethyl Sulfide ($\text{CH}_3\text{-S-CH}_3$)

Dimethyl sulfide is a natural component in milk, influenced by the diet of the cow (8). Although it has been detected in Cheddar cheese by numerous investigators (19, 20), its role in cheese flavor is unclear. It does not consistently occur in the “cheesy” fraction

of Cheddar cheese distillates nor does it correlate with Cheddar cheese flavor development. It can be synthesized by several species of microorganisms in milk.

Methional ($\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CHO}$)

Methional is a volatile sulfur compound implicated in the flavor of Cheddar cheese. It is suggested that a combination of methional, diacetyl, and butyric acid are responsible for a characteristic Cheddar cheese aroma. However, overproduction can lead to the meaty brothy flavors and odors (19). It may add to the flavor of Cheddar cheese via its conversion to methanethiol, either by decarboxylation reactions or by spontaneous degradation.

The mechanism for methional generation in Cheddar cheese has not been elucidated, but it can form via the Strecker degradation of methionine. Strecker degradation is the reaction of an amino acid and a diketone, which results in the formation of an aldehyde one carbon smaller than the original amino acid. Methional formation can also occur via a light-catalyzed reaction between methionine and riboflavin in milk.

S-methylthioesters ($\text{CH}_3\text{-S-CO-R}$)

Fractions contributing to the flavor of several smear-ripened soft cheeses contain S-methyl thioesters with chain lengths ranging from 2-10 carbon atoms. The specific flavors of each thioester depend on their chain length and configuration. Thioesters are synthesized by several lactic acid bacteria, including *Lactococcus lactis*, and some flavor adjunct bacteria, including *Brevibacterium linens*.

Methionine and Cysteine Metabolism

Cysteine and methionine are the sulfur containing amino acids available in casein. In all the caseins in the cheese matrix, there are four residues that have cysteine and there are 16 residues that have methionine (5). This small amount of substrate does not account for production of the total volatile sulfur in cheese.

Early studies show that the cheese flavor and aroma increase during aging with a concomitant increase in methionine content. Wallace and Fox (24) found that after ripening, all the cheese samples increased in the methionine content to 160 mg/kg of cheese except the control (the one without starter bacteria added to it), which contained 70 mg/kg of cheese. This increase in the methionine level is about 10 times higher than that can be accounted for with the number of residues in the casein molecules. These results lead to the suggestion that an additional mechanism, presumably due to bacterial metabolism, is working in cheese that results in additional methionine production from a non-casein source.

Many bacteria commonly found in cheese make use of methionine to give rise to VSC (26). Studies using ^{35}S -methionine and ^{13}C -methionine indicate that methionine is a precursor of methanethiol and other volatile sulfur compounds. Although the catabolic pathway for methionine in lactococci is not completely delineated, possible pathways involve transaminases, methionine γ -lyase (EC 4.4.1.11), and cystathionine γ -lyase (EC 4.4.1.1) or cystathionine β -lyase (EC 4.4.1.8) (5) (Fig. 1).

Aminotransferases in general have broadly overlapping substrate specificities and can catalyze reversible transamination reactions. Aminotransferases are capable of

transferring the α -amino group of amino acids to a α -keto acid acceptor and have been studied in several microorganisms. Aminotransferases capable of the transamination of aromatic amino acids have been described in lactococci. Aromatic amino acid aminotransferases are also capable of the transamination of methionine, which results in the formation of keto-methyl thiobutyrate (KMTB), which can be degraded both enzymatically and nonenzymatically to form methanethiol (10). Aromatic aminotransferases in lactococci exhibit substantial transaminase activity using methionine as a substrate (11, 27), as does the branched chain aminotransferase (22). Inactivation of the aromatic aminotransferase leads to a 50% reduction in methionine degradation (22). The total aminotransferase activity is inhibited by increasing levels of methionine in the growth media in lactococci and some lactobacilli (4). However, in *B. linens* addition of methionine and methionine-containing peptides increases aminotransferase activity (4). Kuiper's group (6, 7) demonstrated that the *metC* gene, which encodes cystathionine β -lyase (CBL), is cotranscribed with the *cysK* gene encoding cysteine synthase because they are together in an operon. Expression is negatively affected by high concentrations of cysteine, methionine, and glutathione in the culture medium, while sulfur limitation increased the level of expression.

Methionine γ -lyase (MGL) directly deaminates and decarboxylates methionine, producing methanethiol, α -ketobutyrate and ammonia. Dias and Weimer (4) partially purified this enzyme from *B. linens* BL2 to homogeneity and found it be active in the typical pH, temperature and salt concentration of ripening Cheddar cheese and in Cheddar cheese slurries. The addition of methionine to the growth medium increased the methionine γ -lyase activity of *B. linens* BL2 (25), in contrast to lactococci.

It has been proposed (1) that cystathionine β -lyase and cystathionine γ -lyase (3) together from lactococci are responsible for cystathionine lyase activity in cheese-like conditions producing methanethiol, α -ketobutyrate and ammonia from methionine. The *metC* gene is responsible for cystathionine lyase activity as disruption of *metC* results in about 60% reduction of cystathionine lyase activity (6). Furthermore, *metC* and *cysK* form a single operon, indicating a coordinated synthesis of at least a part of both biosynthetic pathways. Methionine increases cystathionine lyase and cysteine synthase activities through the regulation of the *metC-cysK* operon. Expression of the *metC-cysK* operon is induced by sulfur limitation and repressed with methionine addition (7).

In light of the production of methionine during aging, the inhibition of catabolic pathways for methionine in lactococci and the production of volatile sulfur compounds, it is reasonable to hypothesize that alternative mechanisms for methionine catabolism are yet to be discovered in lactococci. Taking these observations together creates conflicting mechanisms and suggests that an undiscovered pathway for production of volatile sulfur compounds from methionine may also exist. Potential sources for the sulfur atom are protein recycling in the cell or acquisition from the environment via sulfur fixation. As noted above, sulfur metabolism is a highly regulated event because acquisition of the sulfur atoms a key aspect to metabolism and energy in microbes (16). Protein recycling is a likely mechanism and will depend on the specific environmental conditions and stress status of the cell. Sulfur fixation in lactococci is unknown, but it is common in other organisms such as *E. coli* and *Pseudomonas aeruginosa* (16).

Bacterial Sulfur Fixation

Sulfur fixation occurs in many bacteria (16). This process starts with the transport of sulfur, often as sulfate (SO_4^{-2}), across the cell membrane (Fig. 2). Subsequently, it is activated to adenosine-5'-phosphosulfate (APS), to 3'-phosphoadenosine-5'-phosphosulfate (PAPS), reduced to sulfite and then to sulfide. It is then transferred into organic compounds like cysteine, methione and eventually into sulfur containing proteins inside the cell. This pathway of sulfate fixation is generally induced when the growth environment is devoid of organic sulfur source. This sulfur-starvation induces of about 20 proteins including transport proteins specific for sulfur, desulfurization enzymes, amino acid transport proteins, sulfur-scavenging proteins, antioxidant proteins and the enzymes in the pathways that lead to cysteine and methionine production in *E. coli* and pseudomonads (17).

Studies with *Pseudomonas aeruginosa* examined the influence of sulfate, sulfite, and sulfide on the expression of sulfhydrylases. Addition of acetylserine and sulfate induced the expression of sulfhydrylases, but sulfite and sulfide showed no effect on its expression. Inducible metabolism of sulfur is wide spread in bacteria (17).

The ability of lactococci to fix sulfur and its application in fermented dairy products is unknown. However, there is free sulfate in milk at approximately 40 mg/L (15). Distribution of organic sulfur in milk is about 20 mg/L in water-soluble organic form, ~200 mg/L in casein and none in lipids leading to a total sulfur content of about 260 mg/L (14).

The level of sulfate in cheese is unknown. It can be assumed that sulfur starvation is likely during cheese ripening with this low starting level and the solubility of sulfate in water (whey). Hence, it seems logical that as cheese ages the cells are starved for sulfur, which may induce expression of involved in sulfate fixation. Perhaps this is an explanation for additional sulfur containing compounds in cheese than can be attributed to amino acids in casein.

Sulfur Metabolism Genes in Lactococci

By examining the genome sequence of *L. lactis* IL1403 (2), we found eight genes that are directly related to sulfur metabolism (Table 2). In addition, seven more genes are related to sulfur metabolism after fixation. One gene (*glyA*) for a sulfur-containing carbohydrate was located, suggesting that lactococci can metabolize these types of sugars to gain access to sulfur. While IL1403 did not contain the entire set of genes to fix sulfate via known pathways in *E.coli*. or *Pseudomonas aeruginosa*, enzymes to fix sulfur via acetyl- or succinyl-derivatives were found. With these observations in mind, we hypothesize that lactococci fix sulfate to produce volatile sulfur compounds via an undefined mechanism during cheese ripening.

The three strains *Lactococcus lactis* spp. *lactis* ML3, *Lactococcus lactis* spp. *lactis* IL1403, *Lactococcus lactis* spp. *cremoris* SK11 were selected for all investigations. ML3 and SK11 are used in cheese industry. IL1403 is a purely laboratory strain. Again we already have complete annotated genome of IL1403, and the genome of SK11 is currently being annotated. Hence, by selecting these strains for our study, we could be in a position to extrapolate the experimental observation to industrial condition and predict

its use and we could also do functional genomic study of sulfate metabolism in lactococci.

TABLE 2. Sulfur fixation related genes found in the *L. lactis* IL1403 genome.

Gene	Enzyme produced by the gene
<i>metA</i>	Homoserine O-succinyltransferase
<i>metB1</i>	Cystathionine γ -synthase
<i>metB2</i>	Cystathionine γ -synthase
<i>cysD</i>	O-acetylhomoserine sulfhydrylase
<i>cysE</i>	Serine acetyltransferase
<i>cysK</i>	Cysteine synthase
<i>cysM</i>	Cysteine synthase
<i>yafB</i>	Sulfate transporter

Hypothesis and Objectives

Hypothesis

Lactococci fix sulfur from inorganic sulfate to produce volatile sulfur compounds.

Objectives

1. Determine availability of inorganic sulfate in milk.
2. Determine the effect of environmental pH and cellular growth phase on sulfate transport in lactococci.
3. Determine assimilation of inorganic sulfate into cellular proteins of lactococci.
4. Determine production of VSC from inorganic sulfate by lactococci.

REFERENCES

1. **Alting, A. C., W. J. M. Engels, S. van Schalkwijk, and F. A. Exterkate.** 1995. Purification and characterization of cystathionine γ -lyase from *Lactococcus lactis* subsp. *cremoris* B78 and its possible role in flavor development in cheese. Appl. Environ. Microbiol. **61**:4037-4042.
2. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Research **11**:731-753.
3. **Bruinenberg, P. G., G. deRoo, and G. K. Limsowtin.** 1997. Purification and characterization of cystathionine γ -lyase from *Lactococcus lactis* subsp. *cremoris* SK11: Possible role in flavor compound formation during cheese maturation. Appl. Environ. Microbiol. **63**:561-566.
4. **Dias, B., and B. Weimer.** 1998. Conversion of Met to thiols by lactococci, lactobacilli and brevibacteria. Appl. Environ. Microbiol. **64**:3320-3326.
5. **Dias, B., and B. Weimer.** 1999. Production volatile sulfur compounds in Cheddar cheese slurries. Int. Dairy J. **9**:605-611.
6. **Fernandez, M., W. van Doesburg, G. A. M. Rutten , et al.** 2000. Molecular and functional analyses of the *metC* gene of *Lactococcus lactis*, encoding cystathionine β -lyase. Appl. Environ. Microbiol. **66**:42-48.
7. **Fernandez, M., M. Kleerebezem, O. P. Kuipers, R. J. Siezen, and R. van Kranenburg.** 2002. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. J. Bacteriol. **184**(1):82-90.
8. **Forss, D.A.** 1979. Review of the progress of dairy science : Mechanism of formation of aroma compounds in milk and milk products. J. Dairy Res. **46**: 691-706
9. **Fox, P. F., and J. M Wallace.** 1997. Formation of flavor compounds in cheese. Advances in Applied Microbiology, Adv Appl Microbiol. **45**:17-85
10. **Gao, S., E. S. Mooberry, and J. L. Steele.** 1998. Use of ^{13}C nuclear magnetic resonance and gas chromatography to examine Met catabolism by lactococci. Appl. Environ. Microbiol. **64**:460-4675.

11. **Gao, S., and J. L. Steele.** 1998. Purification and characterization of oligomeric species of an aromatic amino acid aminotransferase from *Lactococcus lactis* subsp. *lactis* S3. *J. Food Biochem.* **22**:197-211.
12. **Grill, H., S. Patton, and J. F. Cone.** 1967. Degradation of ³⁵S-methionine to methyl mercaptan in surface-ripened cheese. *J. Agric. Food Chem.* **15**:392-394.
13. **Grosch, W.** 1994. Determination of potent odourants in foods by aroma extract dilution analysis (AEDA) and calculation of odour activity values (OVAs). *Flavour and Fragrance J.* **9**: 147-158
14. **Jenness, R.; Patton, S.** 1959. The composition of milk. In *Principles of Dairy Chemistry*; 1st ed. John Wiley and Sons, Inc., NY.
15. **Johnson, A.H.** 1974. In *Fundamentals of Dairy Chemistry*, 2nd ed. Webb et al. (ed.), *The Composition of Milk*, p. 21-23. The AVI Publishing Co. Inc., N.Y.
16. **Kertesz, M.** 1999. Riding the sulfur cycle – metabolism of sulfonates and sulfate esters in gram negative bacteria. *FEMS Microbiol. Re.* **24**:135-175.
17. **Kertesz, M., T. Leisinger, and Cook, A.M.** 1993. Protein induced by sulfate limitation in *Escherichia coli*, *Pseudomonas putida*, or *Staphylococcus aureus*. *J. Bacteriol.* **175**:117-1190.
18. **Law, B. A., and M. E. Sharpe.** 1978. Formation of methanethiol by bacteria isolated from raw milk and Cheddar cheese. *J. Dairy Res.* **45**:267-275.
19. **Manning, D.J. and Robinson H.M.** 1973. The analysis of volatile substances associated with Cheddar-cheese aroma. *J. Dairy Res.* **40**: 63-75.
20. **McGuan, W.A.** 1975. Cheddar cheese flavor. A review of current progress. *J. Agric. Food Chem.* **23**: 1047-1050.
21. **Milo, C. and Reineccius, G.A.** 1997. Identification and quantification of potent odorants in regular-fat and low-fat milk Cheddar cheese. *J. Agric. Food Chem.* **45**: 347-352.
22. **Rijnen, L., Bonneau, S. and Yvon, M.** 1999. Genetic characterization of the major lactococcal aromatic aminotransferase and its involvement in conversion of amino acids to aroma compounds. *Appl. Environ. Microbiol.* **65**:4873-4880.
23. **Urbach, G.** 1993. Relations between cheese flavour and chemical composition. *Int. Dairy J.* **3**:389-422.

24. **Wallace, J. M., and P. F. Fox.** 1997. Effect of adding free amino acids to Cheddar cheese curd on proteolysis, flavour and texture development. *Intl. Dairy J.* **7**:157-167.
 25. **Weimer, B., B. Dias, M. Ummadi, J. Broadbent, C. Brennand, J. Jaegi, M. Johnson, F. Milani, J. Steele, and D. V. Sisson.** 1997. Influence of NaCl and pH on intracellular enzymes that influence Cheddar cheese ripening. *Lait.* **77**:383-398.
 26. **Weimer, B., K. Seefeldt, and B. Dias.** 1999. Sulfur metabolism in bacteria associated with cheese. *Antonie van Leeuwenhoek* **76**:247-261.
 27. **Yvon, M., S. Thirouin, L. Rijnen, D. Fromentier, and J. C. Gripon.** 1997. An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. *Appl. Environ. Microbiol.* **63**:414-419.
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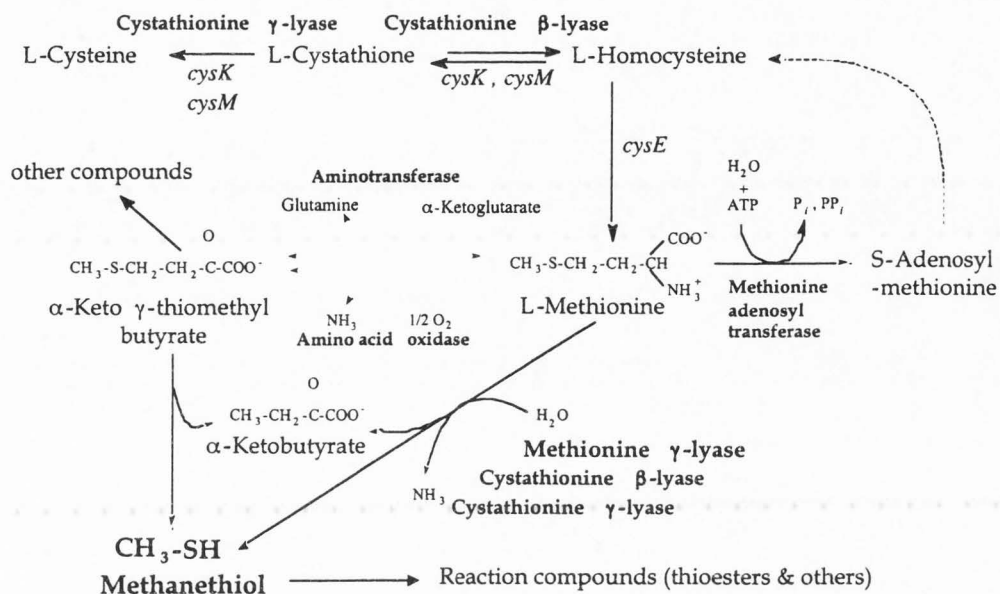


Figure 1. Methionine metabolism pathway for production of volatile sulfur compounds in lactococci with relevant genes and enzymes are shown (*cysK*, *cysM* are gene loci responsible for the enzyme cysteine synthases).

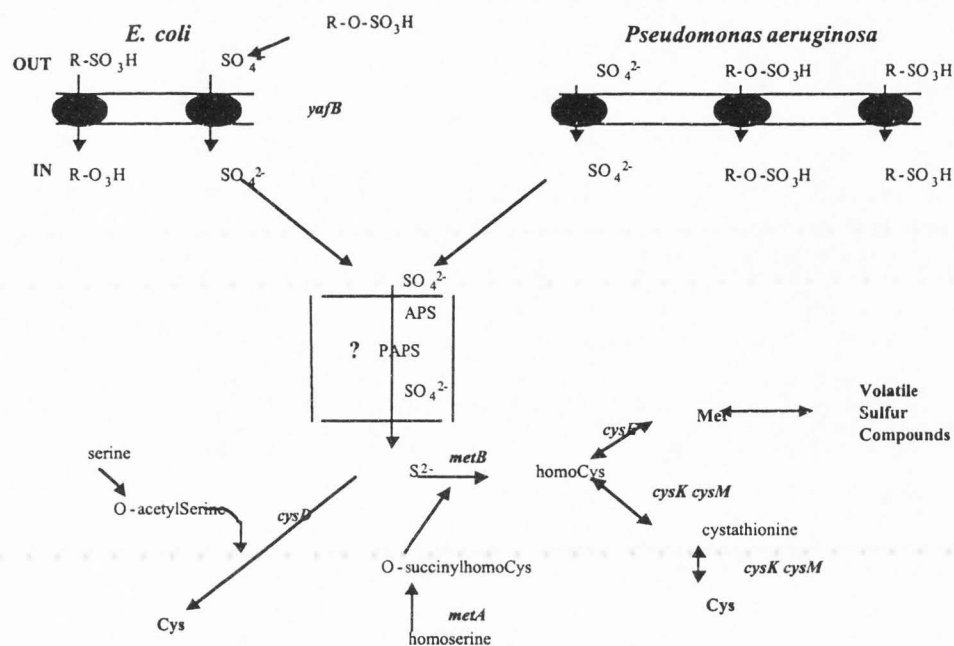


Figure 2. Sulfur cycle in bacteria. Left panel depicts the pathway adopted by *E. coli* for assimilation of sulfate and the right panel depicts the same in *P. aeruginosa*. The genes found in *Lactococci lactis* IL1403 that can give rise to the enzymes responsible for similar reaction catalysis are shown in bold. The boxed section represents the pathway of sulfate assimilation for which no gene has yet been characterized in the lactococcal genome.

CHAPTER II

A TURBIDIMETRIC DETERMINATION OF SULFATE IN MILK

Abstract

Sulfur is a trace element in milk but it plays a significant role in the metabolic pathways in the dairy bacteria used in fermented dairy products manufacture. To study the utilization of sulfate in milk by these bacteria, the measurement of sulfate content in milk is critical. However, no method is available for its determination in high protein, fat, and sugar environments, such as milk. The aim of this work was to design a detection method for the determination of total sulfate content of milk. This was done with addition of barium chloride to whey that resulted in an insoluble barium sulfate precipitate. The concentration was measured via the turbidity (600 nm) of the insoluble barium sulfate salt. The sulfate concentration in milk was determined by comparison with the standard curves of sulfate in Tris and whey from skim and whole milk. The correlation coefficients of the standard curves ranged from r^2 of 0.98 to 0.99 in the three sample types. Sulfate was observed to leach from the GFA filter and taken into consideration in the calculation of actual sulfate content in the samples. This work produced a repeatable and simple method that determined the average sulfate content of skim and whole milk to be ~49.2 ppm and 46.7 ppm, respectively. The limit of detection of the test was ~ 2.5 ppm in Tris and ~10 ppm in both milk types. The repeatability of the method was ~ 0.12%.

Introduction

Flavor is one of the most important attributes of fermented dairy products that lead to its wide acceptance and purchasing decisions among customers. Cheese is one of the most and scientifically investigated attributes of fermented dairy products. Cheese flavor is not due to the presence of a single compound, but is created from a complex, but balanced, mixture of compounds. Specific products of microbial and chemical reactions in the cheese, with specific concentrations, lead to the typical flavor of Cheddar cheese (1 - 6). Sulfur-containing compounds comprise a distinct and desirable fraction of Cheddar cheese flavor and aroma (7 - 11). The total volatile sulfur compounds produced in cheese during ripening is more than the equivalent amount to the original total organic sulfur-containing compounds naturally present in the milk from which the cheese is manufactured (12). Presumably, inorganic sulfate in milk provides the additional sulfur atom.

A method to determine the sulfate content in milk was not available; however, a method used for sulfate assessment in water is well defined (13). The influence of the protein, fat, and carbohydrate in milk was unknown. The aim of this work was to develop a simple method to determine total sulfate content in milk. The basis of this technique relies on precipitation of barium sulfate upon addition of barium chloride to whey.

Chemicals and Solutions

Cupric sulfate (CuSO_4), barium chloride (BaCl_2) crystals and Tris were purchased from Fisher Scientific (Fair Lawn, NJ). A hydrochloric acid solution (1 M) and 0.5 mM Tris (pH 7.2) were made in double deionized water. GFA filters and Whatman filter #1 were from Whatman International Ltd (Maidstone, England) and purchased from Fisher Scientific.

Instruments

An Accumate^R pH meter (Denver Instrument Company, Colorado) and a SPECTRONIC 20D⁺ spectrometer (Thermo Spectronic Inc., NY) were used.

Milk Sample Preparation

Milk samples were purchased from seven different local grocery stores. Representative samples (100 ml) were transferred to clean conical flasks. Whole milk samples were cooled to 4°C and gravity filtered through GFA to remove the fat. The filtrate was collected and brought to room temperature before addition of 1 M HCl to a pH of 4.8. Whey was collected by gravity-filtration through Whatman #1 filter paper. The absorbance of this whey was measured at 600 nm (A_{600}) using a SPECTRONIC 20D⁺ (Thermo Spectronic Inc., NY) as the blank. Then BaCl_2 crystals were added to the same whey at 50,000 ppm. The mixture was stirred thoroughly before the absorbance at 600 nm (A_{600}) was measured using a SPECTRONIC 20D⁺ (Thermo Spectronic Inc., NY).

Sulfate Determination

Standard curves of the sulfate concentration were generated in Tris, skim and whole milk by adding 10 ppm to 120 ppm amounts of CuSO_4 . Commercial milk samples were prepared as described above to determine the sulfate content via absorbance. This value was used to determine the total sulfate concentration using the regression equations obtained from the best-fit standard curves of skim and whole milk.

When we added increasing levels of sulfate (0 to 120 ppm) extraneously to skim and whole milk and measured the turbidity of barium sulfate formed upon addition of barium chloride at the rate of 50,000 ppm, we found that the turbidity was linearly related to the sulfate concentration with an r^2 value of 0.98. Hence the relationship between sulfate and turbidity can be represented by the equation: $y = m(x + k) + c$ [Eq. 1]; where, y represents the turbidity at 600nm, x represents the added sulfate concentration, and k represents the native sulfate content of the milk sample. Now when we put any set of corresponding values of added sulfate and the turbidity in the equation (1), we can get the value of k , which is the native sulfate content of the milk sample as elaborated below. Suppose, for one skim milk sample we measured the turbidity as 0.85 when we added 80 ppm sulfate extraneously to it. If the values of x and y are inserted into equation (1) the equation becomes, $0.85 = m(80+k) + c$ [Eq. 2].

The values of m and c in the case of skim milk (Figure 2B) are 0.011 and 0.463, respectively. When we put these values in Eq. 2, we can calculate the value of k as 39.36, which is the native sulfate content in the skim milk sample.

Sulfate Leaching from GFA filters

Sulfate leaching was determined from three different production lots of Whatman GFA filters by measuring the A_{600} after multiple 25-ml increments of Tris buffer passed through the membrane to a total volume of 200 mL. The concentration of sulfate was determined with the standard curve in Tris. The leaching was determined with three replications from each production lot and the result was reported as the average leaching between the replications and the lots.

Statistics

The standard curves were done with 11 replications and the average was used in the regression for the estimation of the concentration. The sulfate concentration was determined in each milk sample with three replications and the values were reported as the mean and standard deviation. Significant differences between the commercial milk sources and the milk types (skim or whole) were calculated as a randomized ANOVA using $\alpha = 0.05$.

Results and Discussion

Optimization of BaCl_2 concentration

To ensure that the BaCl_2 concentration was not limiting the interaction with sulfate (held at 120 ppm), different levels were added to milk (Figure 3A). The absorbance increased linearly to 20,000 ppm of BaCl_2 . Further increases of BaCl_2 to 100,000 ppm did not increase the signal. Therefore, further work used 50,000 ppm to ensure excess barium to force precipitation of barium sulfate. This concentration of

BaCl₂ addition to milk was judged to be adequate since milk was reported to contain ~40 ppm of sulfate (11).

GFA Leaching

During whole milk sample preparation, we observed that the GFA filter leached sulfate into the whey. To determine the extent of leaching, the GFA membranes were washed with 0.5 mM Tris (pH 7.2) and the sulfate content was determined over the volume added. Three different lots of membranes were washed with incremental addition of 200 mL of Tris to elute any residual sulfate from the membrane filter. A background concentration of sulfate was determined in washings.

Decreasing amounts of sulfate leached from the membrane with increasing washing (Figure 3B). Approximately seven 25-mL aliquots were needed to remove the leaching sulfate from the filters. No significant difference was observed between the three membrane lots for sulfate leaching. As a consequence of this observation and prior to sulfate determination in milk, each GFA membrane was washed with 200 mL of 0.5 mM Tris (pH 7.2) to remove residual sulfate.

Sulfate Standards

Standard curves for sulfate concentration were determined in 50 mM Tris (pH 7.2), skim milk and whole milk (Figure 4). The linear region for sulfate determination in 0.05 mM Tris (Figure 4A) was between 5 and 50 ppm ($r^2 = 0.98$). This result is comparable to that found in water using this method (13).

We assumed that milk contained sulfate when the standard curve was determined and was used as the basis to determine the linear range (Figure 4B and C). When we

added sulfate to the milk sample and measured the turbidity of the insoluble barium sulfate salt, we plotted the absorbance reading against the added sulfate concentration in milk. In skim and whole milk the linear range was at least 120 ppm ($r^2 = 0.99$). Higher sulfate levels were not investigated. These curves were used to determine the sulfate concentration in commercial milk samples.

The average repeatability of the standard curve determination ($n = 11$) was determined to be 5.2% in 50 mM Tris, 0.65% in skim milk and 1.07% in whole milk at concentrations above 10 ppm.

Sulfate Content in Milk

The total sulfate content was significantly different in milk samples from the different commercial sources ($p < 0.0001$) (Table 1). Using the Fisher's Least Significant Difference test, commercial sources 1, 2, and 4 differed significantly ($LSD = 1.7$) from sources 3 and 4 (Figure 5A). While the statistical analysis indicated significance, the practical difference between the commercial source groups was small, 5.5 ± 0.09 ppm ($\sim 10\%$), suggesting that the practical use of this information to milk processing has limited value. Presumably, feed type, variety of cow, lactation stage, number of lactations, collection regime, and bacterial growth in the various milk sources are involved in this difference (14). The practical implication of this difference is unknown and beyond the focus of this work. Therefore, the mechanisms leading to these differences were not explored further in this work.

The sulfate content was significantly higher in skim compared to whole milk ($p < 0.0001$) at 39.8 ± 0.07 ppm and 36.2 ± 0.07 ppm, respectively. While this is a 9.04%

difference, the total concentration difference was small in practical terms, 3.6 ppm.

These values were similar to the previously published value of 40 ppm (11) in skim milk, indicating this method was adequate to determine the total sulfate content in milk.

The difference between skim and whole milk may be due to fat removal during sample processing. While this is possible, the practically small difference in the total sulfate concentration, we suspect this to be unlikely. If the sulfate content changed due to the fat removal, the sulfate must be bound to the fat globules or glycerides. This is also unlikely since each is negatively charged at the pH of milk. It is also unlikely that the sulfate was removed by the GFA filtration step since GFA leached sulfate. In each milk type the concentration was well below the upper limit of the standard curve, even with residual sulfate in milk, making it is unlikely that the barium concentration was limiting in whole milk. While many explanations are possible to describe the difference between skim and whole milk, none of them were explored because the practical difference was small.

Limit of Detection

The minimum limit of detection (LOD) was defined to be three standard deviations greater than the background. Using this criterion, the LOD in 50 mM Tris (pH 7.2) was 5.6 ppm, 5.9 ppm in skim milk, and 11.7 ppm in whole milk. The amount of sulfate found in the unknown milk samples was well within the range of detection for milk.

Considering all the data, this method was a simple and repeatable method that accurately determined the total sulfate concentration in milk. Using this method we found

the sulfate contents of skim and whole milk as 49.2 and 46.7 ppm, respectively. Sulfate determination in unknown milk samples were done in triplicate with an RSD_r of 0.12%.

This method had adequate repeatability for further use in milk.

References

1. Fernandez, M.; Doesburg, W. van; Rutten, G. A. M.; et al. Molecular and functional analyses of the *metC* gene of *Lactococcus lactis*, encoding cystathionine β -lyase. Appl. Environ. Microbiol. **2000**, 66, 42-48.
2. Fernandez, M.; Kleerebezem, M.; Kuipers, O. P.; Siezen, R. J.; and Kranenburg, R. van. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. J. Bacteriol. **2002**, 184(1), 82-90.
3. Law, B. A.; and Sharpe, M. E. Formation of methanethiol by bacteria isolated from raw milk and Cheddar cheese. J. Dairy Res. **1978**, 45, 267-275.
4. Seefeldt, K.; Weimer, B. C. Diversity of sulfur compounds in lactic acid bacteria. J. Dairy Sci. **2000**, 83, 2740-2746.
5. Urbach, G. Relations between cheese flavour and chemical composition. Int. Dairy J. **1993**, 3, 389-422.
6. Weimer, B.; Seefeldt, K.; and Dias, B. Sulfur metabolism in bacteria associated with cheese. Antonie van Leeuwenhoek. **1999**, 76, 247-261.
7. Adams, M. W. W.; Holden J.F.; Menon A.L.; et al. Key role for sulfur in peptide metabolism and in regulation of three hydrogenases in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **2001**, 183, 716-724.
8. Bonnarne, P.; Psoni, L.; and H. E. Spinnler. Diversity of L-methionine catabolism pathways in cheese-ripening bacteria. Appl. Environ. Microbiol. **2000**, 66, 514-5517.
9. Chin, H. W.; Lindsey, R. C. Mechanism of formation of volatile sulfur compounds following the action of cysteine sulfoxide lyases. J. Agric. Food Chem. **1994**, 42, 1529-1536.
10. Dias, B.; Weimer, B. Conversion of Met to thiols by lactococci, lactobacilli and brevibacteria. Appl. Environ. Microbiol. **1998**, 64, 3320-3326.

11. Dias, B.; Weimer, B. Production volatile sulfur compounds in Cheddar cheese slurries. *Int. Dairy J.* **1999**, 9, 605-611.
12. Fox, P. F.; and Wallace, J. M. Formation of flavor compounds in cheese. *Advances in Applied Microbiology*, **1997**, 45, 17-85.
13. Sulfate in water. Turbidimetric method. 973.57. In *AOAC Official Methods of Analysis*; 15th Edition; Kenneth, H., Ed; AOAC Inc.: Arlington, Virginia, 1990; pp 330 – 331.
14. Jenness, R.; Patton, S. The Composition of Milk. In *Principles of Dairy Chemistry*; John Wiley and Sons, Inc.: New York, 1959; pp 3.

TABLE 3. ANOVA table of sulfate content variation with commercial sources and fat content.

SV	DF	SS	p-value
Purchase location	4	163.03	<0.0001
Fat content	1	45.93	<0.0001
Location x Fat	4	111.04	<0.0001

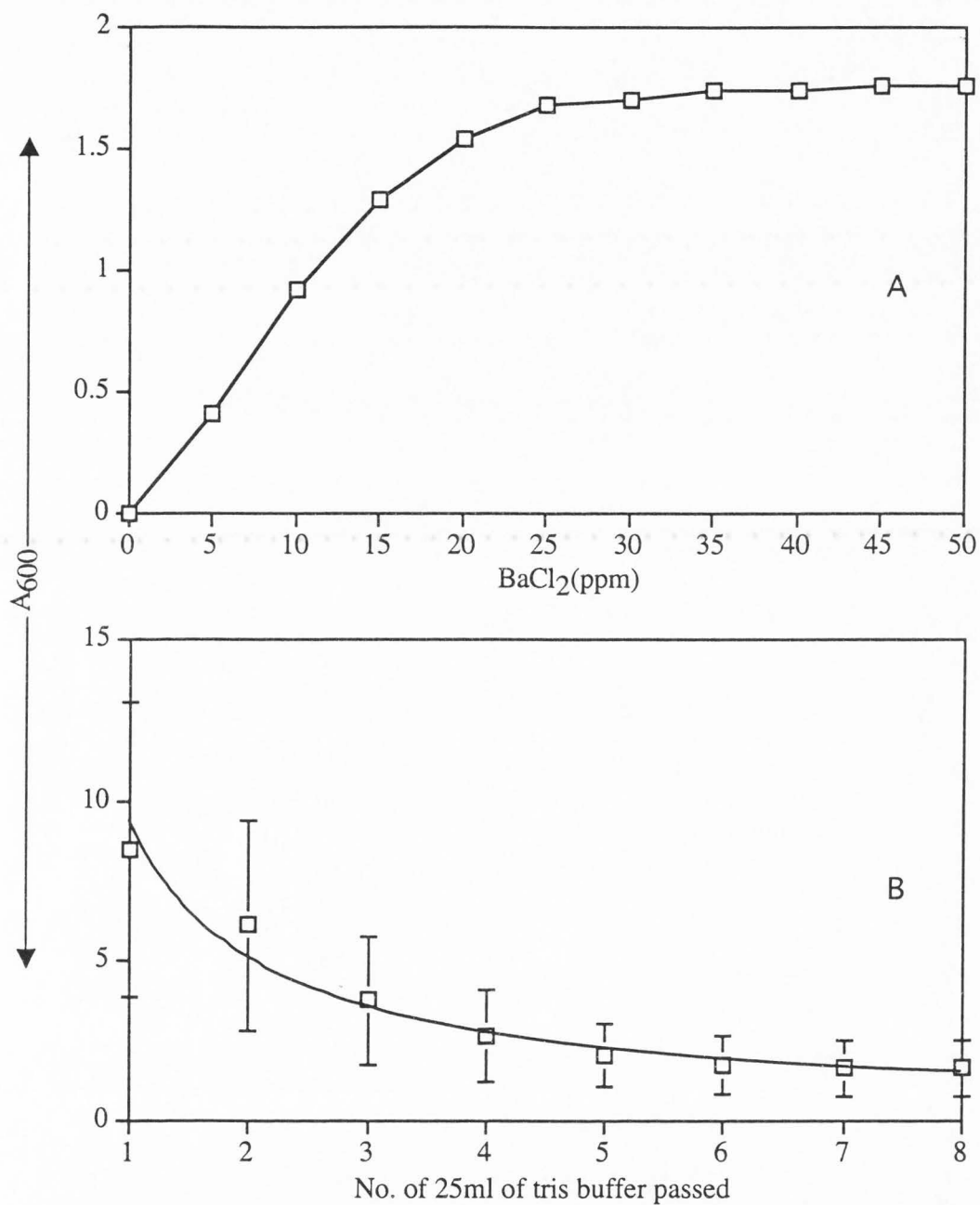


Figure 3. Determination of sulfate with BaCl_2 . Panel A depicts addition of BaCl_2 to 50 mM Tris (pH 7.2) with 120 ppm of sulfate. Panel B depicts sulfate leaching in washings from GFA membranes.

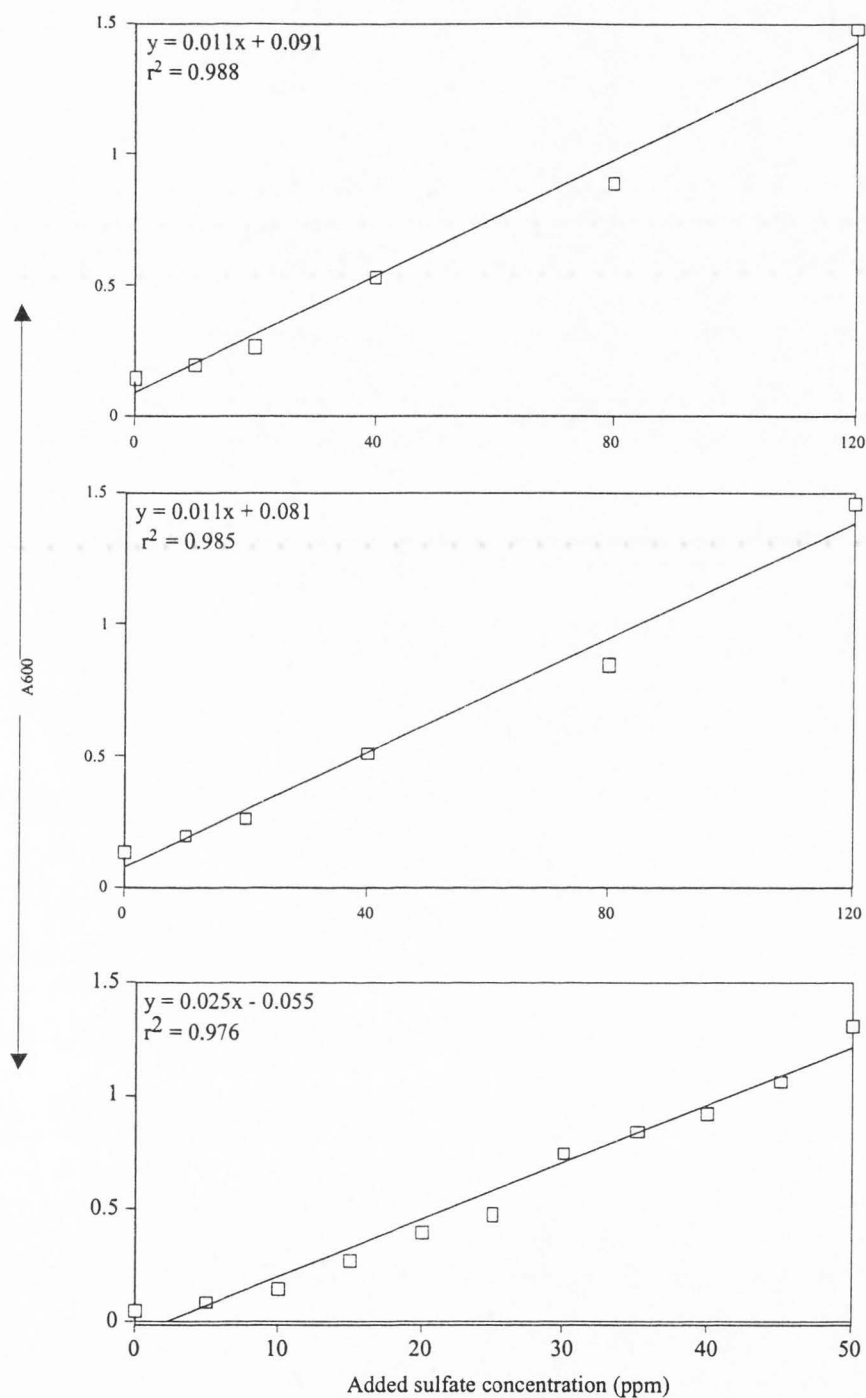


Figure 4. Standard curves of the sulfate content in 50 mM Tris (pH 7.2) (Panel A), skim milk (Panel B), and whole milk (Panel C). All determinations were done with 11 replications. The error bars were plotted, but cannot be seen because they were too small for the y-scale. Linear regression was done to determine the equation and the curve fit for each panel.

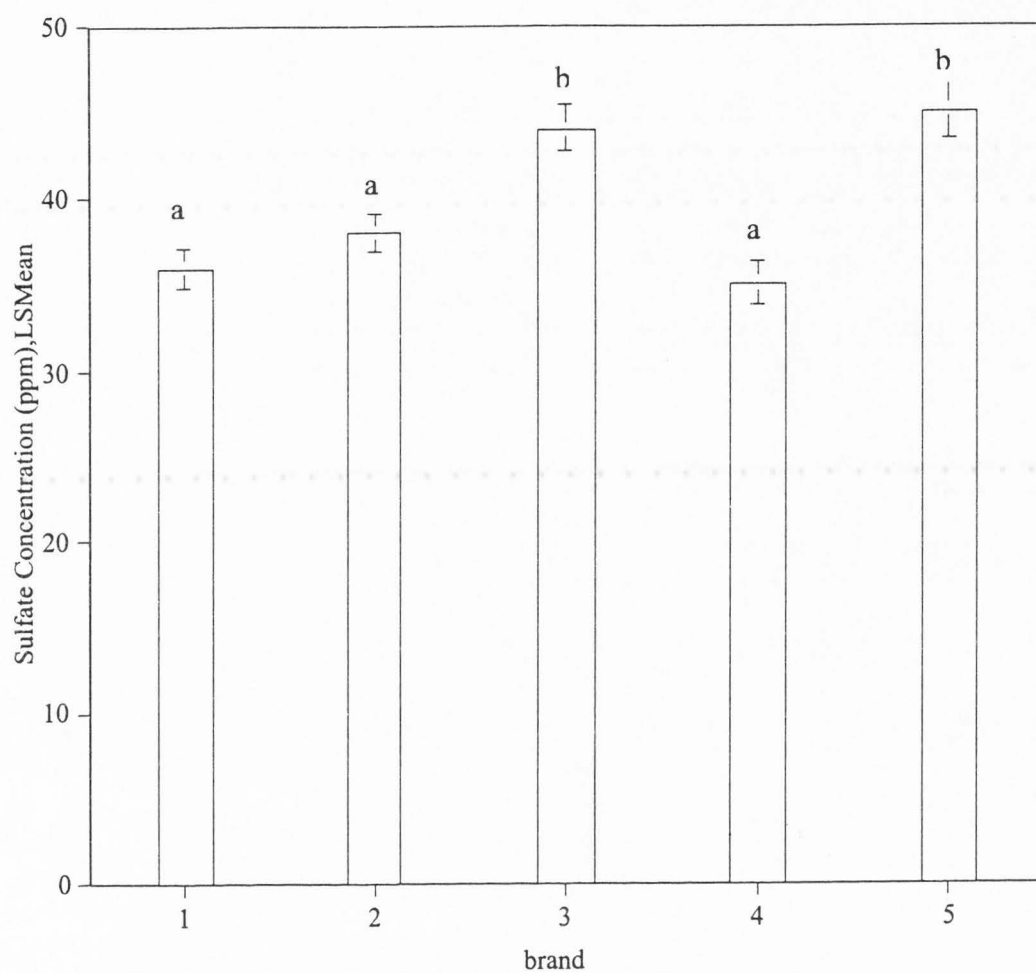


Figure 5. The sulfate content in milk from different commercial sources. Bars with the same letter were not significantly different ($\alpha = 0.01$)

CHAPTER III

SULFATE TRANSPORT AND ITS CONVERSION INTO VOLATILE SULFUR
COMPOUNDS BY LACTOCOCCI

Abstract

In milk, sulfur occurs in low quantities as inorganic sulfate or in casein. It has a significant role in the metabolism of bacteria and is directly related to the product quality. Sulfate metabolism in bacteria associated with the fermented foods is unexplored. In cheese production, especially in Cheddar cheese, a particular balance of volatile sulfur compounds (VSC) contribute positively to the characteristic flavor of the final product. The hypothesis of this work was that sulfate is fixed by lactococci to produce VSCs. Three strains of lactococci transported sulfate in chemically defined medium. Transport increased during acidic conditions and exponential growth. Assimilation of sulfate into cellular protein was not observed using two-dimensional gel electrophoresis, suggesting that the sulfur atom from sulfate was not incorporated into cellular protein. VSCs were found in the headspace of all strains with sulfate as the only sulfur source using GC analysis and radiotracer studies, confirming lactococci fixed sulfur into VSC. The mechanism of fixation and VSC production was not determined.

INTRODUCTION

A particular balance among the products of biochemical and chemical reactions in Cheddar cheese lead to the typical flavor of cheese. Sulfur compounds comprise a distinct fraction of Cheddar cheese flavor and aroma (27). Grill et al. (17), using reagents to trap specific compounds in Cheddar cheese slurries, found that elimination of sulfur compounds adversely affects the aroma of Cheddar cheese (29, 33). However, methanethiol is the only sulfur-containing volatile compound that positively correlates to flavorful Cheddar cheese (23, 28), independent of its non-cheese flavor profile.

Cysteine and methionine are the sulfur-containing amino acids available in casein (20). In all the caseins in the cheese matrix, there are four residues that have cysteine and there are 16 residues that have methionine (10). Wallace and Fox (31) added casein-derived amino acids to cheese during manufacture in an effort to improve volatile sulfur compound production during cheese ripening with a concomitant flavor enhancement. During ripening, the methionine concentration increased to 160 mg/kg, a two-fold increase above the control without starter bacteria added (13). Even the increase in the control is higher than can be contributed by total hydrolysis of all the casein in cheese to release free methionine. Presumably, another sulfur source in milk was utilized by the lactic acid bacteria to generate the additional sulfur-containing amino acids or VSCs in cheese.

One possible source is inorganic sulfate. Sulfate occurs in milk at concentrations between 49 and 47 ppm (Chapter II). The genome of the *Lactococcus lactis* spp. *Lactis* IL1403 contains several genes related to sulfur metabolism: a putative sulfur transporter

(*yafB*) and genes involved in methionine and cysteine metabolism (*metA*, *metB1*, *metB2*, *glyA*, *cysD*, *cysE*, *cysK*, and *cysM*). *E. coli* and *Pseudomonas* also have these genes in addition to other genes that are involved in sulfur fixation into organic sulfur compounds (25, 26). These observations and studies lead us to hypothesize that lactococci transport and fix inorganic sulfur into organic sulfur compounds those are subsequently converted to volatile sulfur compounds (VSC). The hypothesis was tested by measuring sulfate transport, investigating the fate of sulfate during growth and assay, and measuring the VSCs in the cultures headspace with sulfate as the only sulfur source.

The three strains *Lactococcus lactis* spp. *lactis* ML3, *Lactococcus lactis* spp. *lactis* IL1403, *Lactococcus lactis* spp. *cremoris* SK11 were selected for all investigations. ML3 and SK11 are used in cheese industry. IL1403 is a purely laboratory strain. Again we already have complete annotated genome of IL1403, and the genome of SK11 is currently being annotated. Hence, by selecting these strains for our study, we could be in a position to extrapolate the experimental observation to industrial condition and predict its use and we could also do functional genomic study of sulfate metabolism in lactococci.

MATERIALS AND METHODS

Bacterial Strains and Growth

Lactococcus lactis ML3 and *Lactococcus cremoris* SK11, *E. coli* K12 were obtained from the USU collection and *Lactococcus lactis* IL1403 was a gift from Dr. Larry McKay, University of Minnesota.

The bacteria were grown in their respective media overnight inoculated (10%) into sterile 10% non-fat dry milk containing 30% glycerol, and frozen at -70°C in 1.8 mL vials. From the freezer vials, ML3 was grown in Ellikers broth, SK11 was grown in M-17 lactose broth and IL1403 was grown in M-17 glucose broth for 16 hours at 30°C before inoculation into a modified chemically defined medium (CDM) (15) in which all sulfate salts are replaced by the respective chloride salts of the same cation, and sodium sulfate and methionine and cysteine were added to it depending on the experiment treatments. This culture was grown at 30°C for 16 to 24 hrs before use. *E.coli*. K12 was grown from the freezer vial in Luria broth for 16 hours at 30°C in a flask of 10 times the volume of the media with constant shaking at 150 rpm.

Chemicals and Solutions

Sodium sulfate (Na_2SO_4), barium chloride (BaCl_2) crystals, MOPS, MES and Tris were purchased from Fisher Scientific (Fair Lawn, NJ). Kodak X-OMAT AR film was purchased from Eastman Kodak Co. (Rochester, NY). Coomassie blue R-250 was purchased from Bio-Rad (Richmond, CA).

Instruments

An Accumate pH meter (Denver Instruments, Denver, CO), SPECTRONIC 20 D⁺ spectrometer (Thermo Spectronic Inc., New York, NY), BioRad IEF Cell, BioRad Protean II 2D gel Electrophoresis Unit (Richmond, CA), LS 6500 Beckman Multipurpose Scinillation Counter (Beckman Instruments, Inc., Fullerton, CA) and a gas chromatography system (17A ver. 3, Shimadzu Corporation, Columbus, MD) were used.

Sulfate Transport

Sulfate transport was determined by measuring the residual sulfate in the spent medium during growth in different conditions in a chemically defined medium (CDM). After growth from the freezer vial in the respective growth media overnight, the stationary phase cells were washed twice with an equal volume of sterile saline (0.85% NaCl). Some of the overnight grown cells were again inoculated in the same growth media for 4 hours and then washed twice in sterile saline to obtain cells in exponential growth phase. These cells are then inoculated into CDM (buffered at pH 4.8, 5.3, 5.8, 6.3, 6.8 and 7.2 using MOPS and MES within their working buffer ranges) to an optical density of $A_{600} = 0.6$ and incubated at 30°C for 24 hours. Samples of cell suspension (10 mL) were drawn from the culture in 30 min intervals for the initial 2 hours followed by 1 hour intervals during the remaining 24 hours. The same experiment was done with starved ML3 cells, too. All test conditions were done in replicate and the data are reported as an average.

Sulfate Determination in Spent Media

Sulfate was determined as described in Chapter III. Briefly, the drawn samples were centrifuged (4°C at 3,470 x g for 10 minutes) prior to 0.2 µm filter-syringe (Fisher Scientific, Atlanta, GA) filtration to obtain cell-free supernatant in a clean, dry glass test tube. Barium chloride crystals were added to a final concentration of 50000 ppm, mixed thoroughly by vortexing for one minute, and the A_{600} was measured. The standard curves of absorbance reading versus sulfate content in buffered CDM and assay were obtained by adding 0, 20, 40, 80, 120 ppm Na_2SO_4 and measuring the A_{600} as described in Chapter

II. The residual sulfate concentration in the spent medium was determined using the respective standard curve formulae derived from linear regression. The residual sulfate content was plotted against time for each strain and pH to determine the amount of transport. All experiments were done in replicate and analyzed using the ANOVA function with JMP software (SAS Institute Inc., Cary, NC).

Cell pellet radioactivity was determined with ^{35}S -sulfate as the sole sulfur source in CDM at pH 5.3 and 6.8. After 2 hours of incubation at 30°C the cells were collected by centrifugation (4°C, 3470 x g for 10 minutes), washed three times with an equal volume of sterile saline, and resuspended in an liquid scintillation cocktail (Fisher Scientific). The average counts per minute (CPM) over 5 minutes were measured. The recorded CPM values from two replications were used to determine fixation.

Sulfate Assimilation

Cellular protein analysis. Assimilation of ^{35}S -sulfate into bacterial protein was determined using two dimensional gel electrophoresis (18) after assaying the cell with ^{35}S -sulfate as the sole sulfur source and in combination with methionine and cysteine for one generation time. CDM devoid of any sulfur source (10 mL) buffered with MOPS at pH 5.3 and 6.8 was inoculated with a fresh culture standardized to an $A_{600} = 0.6$. Each culture was added with ^{35}S -sulfate (120 ppm) alone, ^{35}S -sulfate (120 ppm) in combination with methionine-cysteine(20 ppm), ^{35}S methionine-cysteine (20 ppm) alone and ^{35}S methionine-cysteine (20 ppm) in combination with non-radioactive sulfate (120 ppm) before analysis with two-dimensional gel electrophoresis.

Prior to analysis, the cells were incubated at 30°C for one generation, centrifuged (room temperature at 3,470 x g for 5 minutes), and the cell pellet was re-suspended in 1 ml of TE buffer (pH 6.8). Sterile glass beads (250 mg) of $\leq 120\mu\text{m}$ diameter (Sigma Chemical Co., St. Louis, MO) were added and vortexed for 10 minutes at highest speed with intermittent chilling on ice to produce a cell free extract. The lysate was centrifuged at (room temperature at 3,470 x g for 5 minutes) before use in two-dimensional analysis.

Two-dimensional gel electrophoresis of total cell lysate supernatant was done as described by Hochstrasser et al. (18) using the BioRad IEF Cell and BioRad PROTEAN II xi 2-D gel electrophoresis unit (Bio-Rad, Richmond, CA), and the gels were stained with Coomassie blue R-250 (Bio-Rad, Richmond, CA), de-stained twice with ethanol-water solution and then dried at room temperature after covering with Biodesign Gel-wrap (Biodesign International, Saco, ME). The dried gel was put into a cassette with Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) and incubated in a dark room at room temperature for 7 d. The exposed film was compared with stained gel spots and the proteins which were expressed and the ones among which had ^{35}S from the various sulfur sources were determined. The same experiment was done with *E. coli* K12 was used as a control in the same experimental conditions to verify the method (Appendix Figure 15).

Volatile sulfur compound analysis. VSCs were determined using gas chromatography as described by Seefeldt and Weimer (27). Prior to GC analysis, each strain was grown from freezer vial as described above and incubated in CDM buffered to pH 5.3 and 6.8 containing 2% sugar (glucose in case of IL1403 and lactose in case of

ML3 and SK11), adjusted to a cell density of $A_{600} = 0.6$, and increasing concentration of sulfate (0, 80, 120 ppm). A control medium that contained sulfate (120 ppm), methionine (20 ppm), and cysteine (20 ppm) were added together was used. An additional control medium lacked sulfate, but contained methionine (20 ppm) and cysteine (20 ppm) was used for comparison of VSC production from organic sulfur.

The cultures were incubated at 30°C for ~16 hours in each medium before analysis of VSC production using a sulfate assay at pH 5.3 or 6.8. After growth in each sulfur-containing medium, the cells were collected by centrifugation ($2,409 \times g$ at 4°C for 10 minutes), washed twice with an equal volume of sterile saline, and re-suspended to an $A_{600} = 0.6$ in the sulfate assay buffer. At pH 5.3 the buffer was MES and at 6.8 the buffer was MOPS containing 120 ppm of sodium sulfate in a headspace vial for GC analysis. Each assay pH was also done with or without the addition of 4% NaCl; thereby, creating four different assay conditions for each growth condition. The headspace vial (22 mL) was sealed with 2 mL of inoculated buffer solutions from each of the four assay conditions and flushed with sterile argon gas for 2 minutes at a pressure of 200 psi and incubated at 30°C for 2 h as described by Dias and Weimer (10).

The VSC analysis was done as described by Dias and Weimer (10) Briefly, the assay headspace vials were loaded onto the autosampler of the Gas Chromatography System (Shimadzu Corporation, Columbus, MD) and equilibrated to 70°C for 15 min before being mixed for 5 minutes at a power setting of 2. A 2 ml injection of the headspace was cryofocused at -70°C onto the top of a SPB1-Sulfur column (30 m, 0.32 mm ID, 100% poly-dimethylpolysiloxane coated, 4µm film thickness, fused silica capillary column; Supelco, Bellefonte, PA) and injected by increasing the temperature to

200°C for 1 min. VSCs were detected using a flame photometric detector, operating in the sulfur mode at 394 nm using a gas chromatography system (17A ver. 3, Shimadzu Corporation, Columbus, MD). The compounds were identified by comparing retention times of known standards.

VSC production by fixation. The samples were prepared as described for GC analysis, except that the sulfur source during the assay was 120 ppm of ^{35}S -sulfate. After the assay incubation, 10 mL of headspace gas was collected in an air-tight volumetric syringe and injected into a scintillation counter vial containing 10 mL of liquid scintillation cocktail (Fisher Scientific). The scintillation vials were mixed for 2 minutes by vortexing at room temperature. The radioactivity of the headspace gas from the culture was measured for 5 minutes using a Beckman Scintillation Counter (LS 6500; Beckman Instruments, Inc., Fullerton, CA). The analyses were done in replicate and the data are reported as an average. Controls that lacked inoculation with cells into the assay were included.

RESULTS

Sulfate Transport

Sulfate transport was determined by measuring residual sulfate in the spent medium during growth at various pH values. ML3 transported significantly ($p < 0.05$) more sulfate than did IL1403 or SK11 at pH 6.3 and below while they were in exponential growth phase. Transport of sulfate by the stationary phase cells of all these three strains were not statistically significantly different. Transport increased significantly

($p < 0.05$) by decreasing the pH of the assay buffer from 7.3 to 4.8 (Figure 6) in both cellular phases.

Sulfate Assimilation

The assay system using ^{35}S -sulfate followed by two-dimensional gel electrophoresis (2D PAGE) was verified using *E. coli* K12 with ^{35}S -sulfate (Appendix 1, Figure 15). Lactococci 2D PAGE patterns were verified using ^{35}S -Met/Cys (Appendix 2, Figures 16-18). Assimilation of sulfate by lactococci was done use this technique. Protein spots were observed in the stained 2D PAGE, but no radiolabeled spots were observed with ^{35}S -sulfate as the sole sulfur source (data not shown). None of the lactococci demonstrated the ability to incorporate sulfur into protein using this technique, despite having radioactivity in the cell pellet. Consequently, each culture was tested for the production of VSCs during growth in various levels of sulfate alone and in combination with organic sulfur sources.

VSC Analysis

Gas chromatography. Prior to assaying the cells with 120 ppm sulfate, cells were grown different levels of sulfate as the sole source of sulfur and in combinations of sulfate with methionine-cysteine to determine interactions of exposure to sulfur sources during growth and the ability to produce VSCs. The VSC production assay conditions were done with 120 ppm of sulfate, two pH and two NaCl conditions. Lack of a sulfur source (sulfur starvation) during growth lead to more VSC production from sulfate in assay compared to addition of any sulfur source. The same observation was made when assayed at pH 6.8 (Figure 7) or pH 5.3 (Figure 8). Addition of NaCl to the assay

condition at pH 6.8 decreased the total VSC production ~2-fold during sulfur starvation (Figure 7B), but had little influence at pH 5.3 (Figure 8B). Interestingly, the least total VSC production was observed when Met and Cys was added.

The primary VSCs produced during the assay were dimethyldisulfide (DMDS) and methylthiobutyrate (MTB). Two unknown compounds with retention times of 3.1 and 4.2 minutes were also observed in some conditions. However, because the cells were exposed to organic sulfur sources before assay with sulfate, it is unclear if the VSCs were produced from the sulfate the assay or from intracellular sulfur sources that could be mobilized during assay. Consequently, this observation was verified using radiolabeled sulfate in the assay followed by scintillation counting of the headspace.

Radioactive VSC production. The initial experiment was done with cells were grown in various concentrations of sulfate with added Met and Cys, as done for GC analysis, followed by assay with 120 ppm of radio-labeled sulfate in the assay at pH 6.8 and 5.3. The headspace from the assay in sulfate was transferred into the scintillation cocktail and the radioactivity was measured to directly assess the radioactive VSC component. In all strains tested, the average CPM recorded from the 15 ml headspace of the assay mixture was 10^5 fold higher than the background or the control lacking cells (Figures 9-11).

To determine the influence of sulfate on the ability of the cells to produce VSC from Met or Cys, the cells were grown in various levels of sulfate and assayed with a mixture of radio-labeled Met and Cys. The radioactive headspace of this assay showed a 2 to 3 fold increase in VSC compared to assay with sulfate (Figures 12-14). These observations confirmed VSC production and established that sulfur starvation during

growth leads to more VSC production. The total radiolabeled volatile sulfur compound production increased with decreasing assay pH, which corresponds to the higher sulfate transport by these bacteria with decrease in pH of the environment (Figure 6). Addition of NaCl had no effect on the corresponding CPM readings in the range from 0 to 4%.

DISCUSSION

Assimilation of sulfate is well known in gram negative bacteria like *E. coli* and *P. aeuginosa* (19, 21, 24, 30, 31, 32), but the sulfate metabolism of lactococci is uncharacterized. However, with the presence of the genes coding for the enzymes responsible for methionine biosynthesis and sulfate transport in the IL1403 genome (2, 3, 7, 11), we hypothesized that lactococci have the potential to fix sulfur into VSCs.

Inspection of the genome sequence found the presence of a sulfate transporter (*yafB*) and other genes associated with methionine and cysteine interconversion (*metA*, *metB1*, *metB2*, *glyA*, *cysD*, *cysE*, *cysK*, *cysM*). Some of these genes are associated with conversion of methionine and cysteine into VSCs (1, 4, 5, 6, 8, 9, 11, 11, 12, 14, 16) in lactococci. This genome contained an entire set of enzymes to fix sulfur via acetyl- or succinyl-derivatives (2, 3). However, these organisms have not been observed to fix sulfur.

To investigate the hypothesis, the total sulfate transport per cell was determined. Each strain increased transport as the pH decreased to 4.8 (Figure 6). This observation indicates that the specific transport of sulfate is likely as suspected by finding the sulfate transporter (*yafB*) in the genome. However, the fate of the sulfate after transport was

unclear. To determine the fate, a proteome analysis was done using 2D PAGE autoradiographs.

This technique has been used to determine sulfur fixation in *E. coli* previously (22) and we suspected that it would be sufficient for our needs. The assay was verified using *E. coli* K12 (Appendix 1) and subsequently used with each strain of lactococci. While the protein stained gels and the methionine/cysteine controls gels produced spots, the 2D PAGE autoradiographs did not contain radiolabeled spots with sulfate as the sole sulfur source. This observation suggested that the cells did not assimilate the transported sulfate into protein. Hence, we measured the cell pellet and found it to be radioactive and speculated that perhaps the sulfate was used to produce VSCs without a large portion being incorporated into protein. This speculation was reasonable and further speculated this observation might be explained by the lack of assay sensitivity to measure the incorporation or that the incorporation rate was too slow to be observed in our conditions (the incorporation was only assessed for 1 generation, ~45 min). The non-incorporation of radioactive sulfur from sulfate into cellular proteins in case of this experiment can be due to the growth of the bacteria in organic sulfur rich media prior to assaying in radiolabeled CDM for 2-D gels. Irrespective of the mechanism, the hypothesis was partially tested by observing transport. The remaining questions were answered by determining influence of growth in different sulfur sources and assaying VSC production with various methods.

VSCs were determined as a function of sulfur source, assay pH and the addition of NaCl to the assay mixture (Figures 7 and 8). VSC production decreased with addition of sulfate or Met/Cys. Addition of Met and Cys agrees with the genetic studies of VSC

production (11, 12) and the addition of Met to cultures and decreasing enzyme activity (10). However, this is the first report of repression with the addition of sulfate.

Due to this unique observation and the possible confounding effect of sulfur mobilization from cellular protein during sulfur starvation (19, 21, 22), we confirmed this observation using radiolabeled sulfate as the sole sulfur source and measured the radioactivity of the headspace of the assay mixture. The growth conditions matched that of the GC analysis and included an extra analysis with methionine/cysteine rather than sulfate. The observation from the GC analysis was confirmed (Figures 9-11) for each strain, demonstrated that the sulfur atom in the VSCs was derived from sulfate in the assay medium. It further verified the repression with addition of sulfate and organic sulfur sources to the growth medium (Figures 12-14). These observations directly prove conversion of sulfate to VSCs and they demonstrate the hypothesis to be true – lactococci fix inorganic sulfur into VSCs. The mechanism for this activity remains to be elucidated.

The biochemical steps for transformation of methionine into various volatile sulfur compounds may be redox regulated reactions. Lactococci do not have the cytochrome system; hence, the use of these redox reactions leading to the formation of VSCs may play a significant role in the maintenance of proton gradient across the cell membrane and thus aids in the survival of the bacteria.

Considering that ~40% of the genome is not characterized for its function, it is reasonable to speculate that yet uncharacterized genes may be associated with sulfur fixation in lactococci. The next step of investigation should be a transcription study of the available whole genome of IL1403 using microarray techniques to determine what genes are involved in sulfur fixation in lactococci.

REFERENCES

1. **Alting, A. C., W. J. M. Engels, S. van Schalkwijk, and F. A. Exterkate.** 1995. Purification and characterization of cystathionine γ -lyase from *Lactococcus lactis* subsp. *cremoris* B78 and its possible role in flavor development in cheese. Appl. Environ. Microbiol. **61**:4037-4042.
2. **Bolotin, A., S. Mauger, K. Malarme, S. D. Ehrlich, and A. Sorokin.** 1999. Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. Antonie van Leeuwenhoek. **76**:27-76.
3. **Bolotin, A., P. Wincker, S. Mauger, O. Jaillon, K. Malarme, J. Weissenbach, S. D. Ehrlich, and A. Sorokin.** 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. Genome Research **11**:731-753.
4. **Bonnarme, P., L. Psoni, and H. E. Spinnler.** 2000. Diversity of L-methionine catabolism pathways in cheese-ripening bacteria. Appl. Environ. Microbiol. **66**:514-5517.
5. **Bruinenberg, P. G., G. deRoo, and G. K. Limsowtin.** 1997. Purification and characterization of cystathionine γ -lyase from *Lactococcus lactis* subsp. *cremoris* SK11: Possible role in flavor compound formation during cheese maturation. Appl. Environ. Microbiol. **63**:561-566.
6. **Collin, J. C., and B. A. Law.** 1989. Isolation and characterization of the L-methionine γ -demethylase from *Brevibacterium linens* NCDO 739. Sci. Aliments. **9**:805-812.
7. **Deguchi, Y., and T. Morishita.** 1992. Nutritional requirements in multiple auxotrophic lactic acid bacteria: Genetic lesions affecting amino acid biosynthesis pathways in *Lactococcus lactis*, *Enterococcus faecium*, and *Pediococcus acidilactic*. Biosci. Biotech. Biochem. **56**:913-918.
8. **Dias, B., and B. Weimer.** 1998. Conversion of Met to thiols by lactococci, lactobacilli and brevibacteria. Appl. Environ. Microbiol. **64**:3320-3326.
9. **Dias, B., and B. Weimer.** 1998. Purification and characterization of L-methionine γ -lyase from *Brevibacterium linens* BL2. Appl. Environ. Microbiol. **64**:3327-3331.
10. **Dias, B., and B. Weimer.** 1999. Production volatile sulfur compounds in Cheddar cheese slurries. Int. Dairy J. **9**:605-611.

11. **Fernandez, M., W. van Doesburg, G. A. M. Rutten, J. D. Marugg, A. C. Alting, R. van Kranenburg, and O. P. Kuipers.** 2000. Molecular and functional analyses of the *metC* gene of *Lactococcus lactis*, encoding cystathionine β -lyase. *Appl. Environ. Microbiol.* **66**:42-48
12. **Fernandez, M., M. Kleerebezem, O. P. Kuipers, R. J. Siezen, and R. van Kranenburg.** 2002. Regulation of the *metC-cysK* operon, involved in sulfur metabolism in *Lactococcus lactis*. *J. Bacteriol.* **184**(1): 82-90
13. **Fox, P. F., and J. M. Wallace.** 1997. Formation of flavor compounds in cheese. *Advances in Applied Microbiology*, **45**: 17-85
14. **Friedrich, C. G., D. Rother, F. Bardischewsky, A. Quentmeier, and J. Fischer.** 2001. Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? *Appl. Environ. Microbiol.* **67**:2873-2882
15. **Gao, S., D. H. Oh, Broadbent, J. R., et al.** 1997. Aromatic amino acid catabolism by lactococci. *Lait.* **77**:371-381
16. **Gao, S., E. S. Mooberry, and J. L. Steele.** 1998. Use of ^{13}C nuclear magnetic resonance and gas chromatography to examine Met catabolism by lactococci. *Appl. Environ. Microbiol.* **64**:460-4675
17. **Grill, H., S. Patton, and J. F. Cone.** 1967. Degradation of ^{35}S -methionine to methyl mercaptan in surface-ripened cheese. *J. Agric. Food Chem.* **15**:392-394
18. **Hochstrasser, D.F., M.G. Harrington, A.C.Hochstrasser, M.J. Miller, and C.R. Merrill.** 1988. Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* **173**:424-435
19. **Hummerjohann, J., E. Kuttel, M. Quadroni, J. Ragaller, T. Leisinger, and M. Kertesz.** 1998. Regulation of the sulfate starvation response in *Pseudomonas aeruginosa*: role of cysteine biosynthetic intermediates. *Microbiology.* **144**:1375-1386
20. **Jenness, R. and Patton, S.** 1959. The Composition of Milk, p. 3. In Jenness, R. and Patton, S. (ed), *Principles of Dairy Chemistry*; John Wiley and Sons, Inc., NY.
21. **Kertesz, M.** 1999. Riding the sulfur cycle – metabolism of sulfonates and sulfate esters in gram-negative bacteria. *FEMS Microbiol. Re.* **24**:135-175
22. **Kertesz, M., T. Leisinger, and A. M. Cook.** 1993. Protein induced by sulfate limitation in *Escherichia coli*, *Pseudomonas putida*, or *Staphylococcus aureus*. *J. Bacteriol.* **175**:117-1190

23. **Law, B. A., and M. E. Sharpe.** 1978. Formation of methanethiol by bacteria isolated from raw milk and Cheddar cheese. *J. Dairy Res.* **45**:267-275
24. **Overmann, J., and H. van Gernerden.** 2000. Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. *FEMS Microbiol. Rev.* **24**:591-599
25. **Ozawa, K., T. Meikari, K. Motohashi, M. Yoshida, and H. Akutsu.** 2000. Evidence for the presence of an F-type ATP synthase involved in sulfate respiration in *Desulfovibrio vulgaris*. *J. Bacteriol.* **182**: 2200-2206
26. **Ploeg, J. R., et al.** 1996. Identification of sulfate starvation-regulated genes in *Escherichia coli*: a gene cluster involved in the utilization of taurine as a sulfur source. *J. Bacteriol.* **178**: 5438-5446
27. **Seefeldt, K. and B. C. Weimer.** 2000. Diversity of sulfur compounds in lactic acid bacteria. *J. Dairy Sci.* **83**:2740-2746
28. **Sharpe, M. E., B. A. Law, and B. A. Philips.** 1977. Methanethiol production by coryneform bacteria: Strains from dairy and human skin sources and *Brevibacterium linens*. *J. Gen. Microbio.* **101**:345-349
29. **Urbach, G.** 1993. Relations between cheese flavour and chemical composition. *Int. Dairy J.* **3**:389-422
30. **Vermeij, P., and M. A. Kertesz.** 1999. Pathways of assimilative sulfur metabolism in *Pseudomonas putida*. *J. Bacteriol.* **181**:5833-5837
31. **Wallace, J. M., and P. F. Fox.** 1997. Effect of adding free amino acids to Cheddar cheese curd on proteolysis, flavour and texture development. *Intl. Dairy J.* **7**:157-167
32. **Weimer, B., K. Seefeldt, and B. Dias.** 1999. Sulfur metabolism in bacteria associated with cheese. *Antonie van Leeuwenhoek.* **76**:247-261
33. **Yvon, M., S. Thirouin, L. Rijnen, D. Fromentier, and J. C. Gripon.** 1997. An aminotransferase from *Lactococcus lactis* initiates conversion of amino acids to cheese flavor compounds. *Appl. Environ. Microbiol.* **63**:414-419

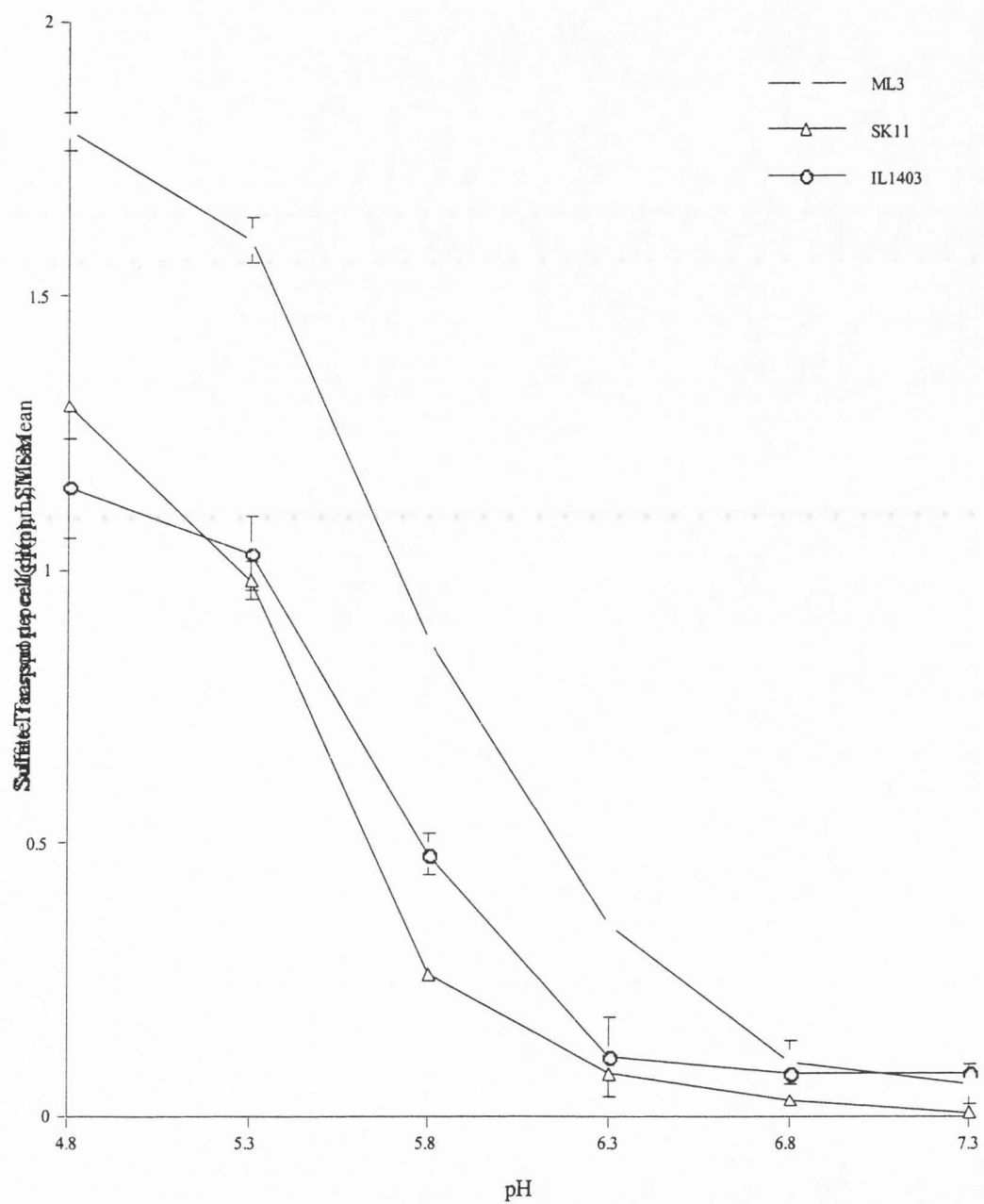


Figure 6. Sulfate transport by lactococci at different pHs. Error bars are standard error of the mean.

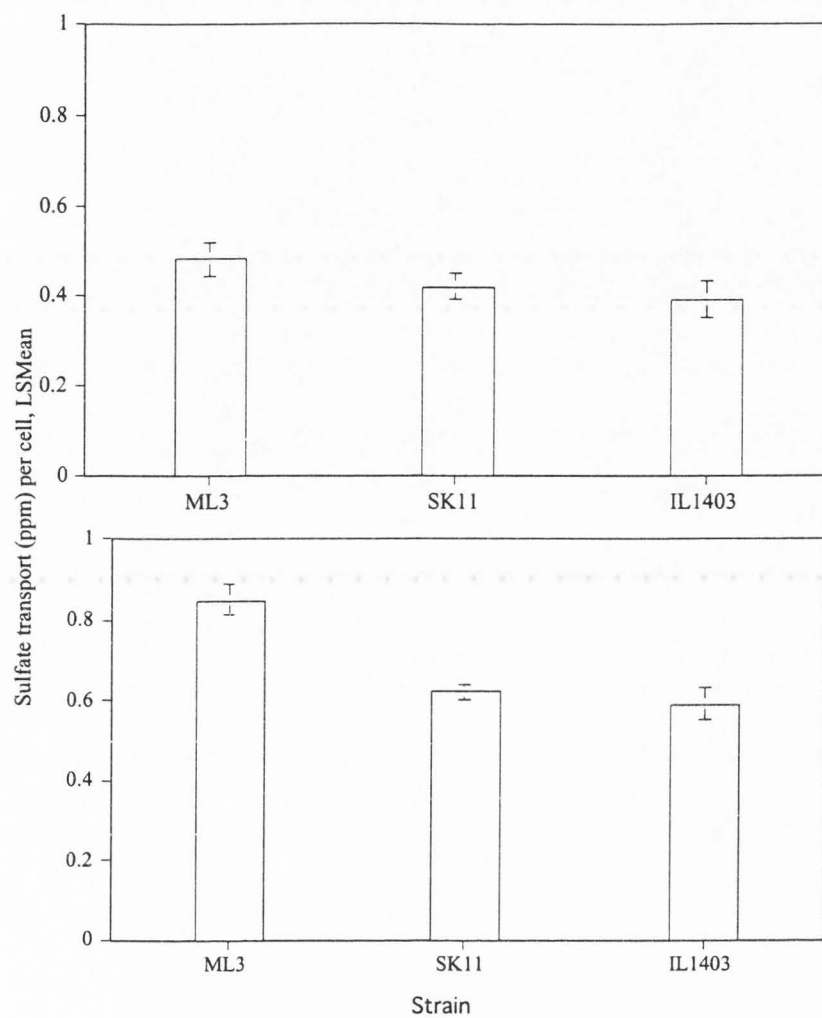


Figure 7. Sulfate transport variation with cellular growth phase in ML3, SK11 and IL1403.

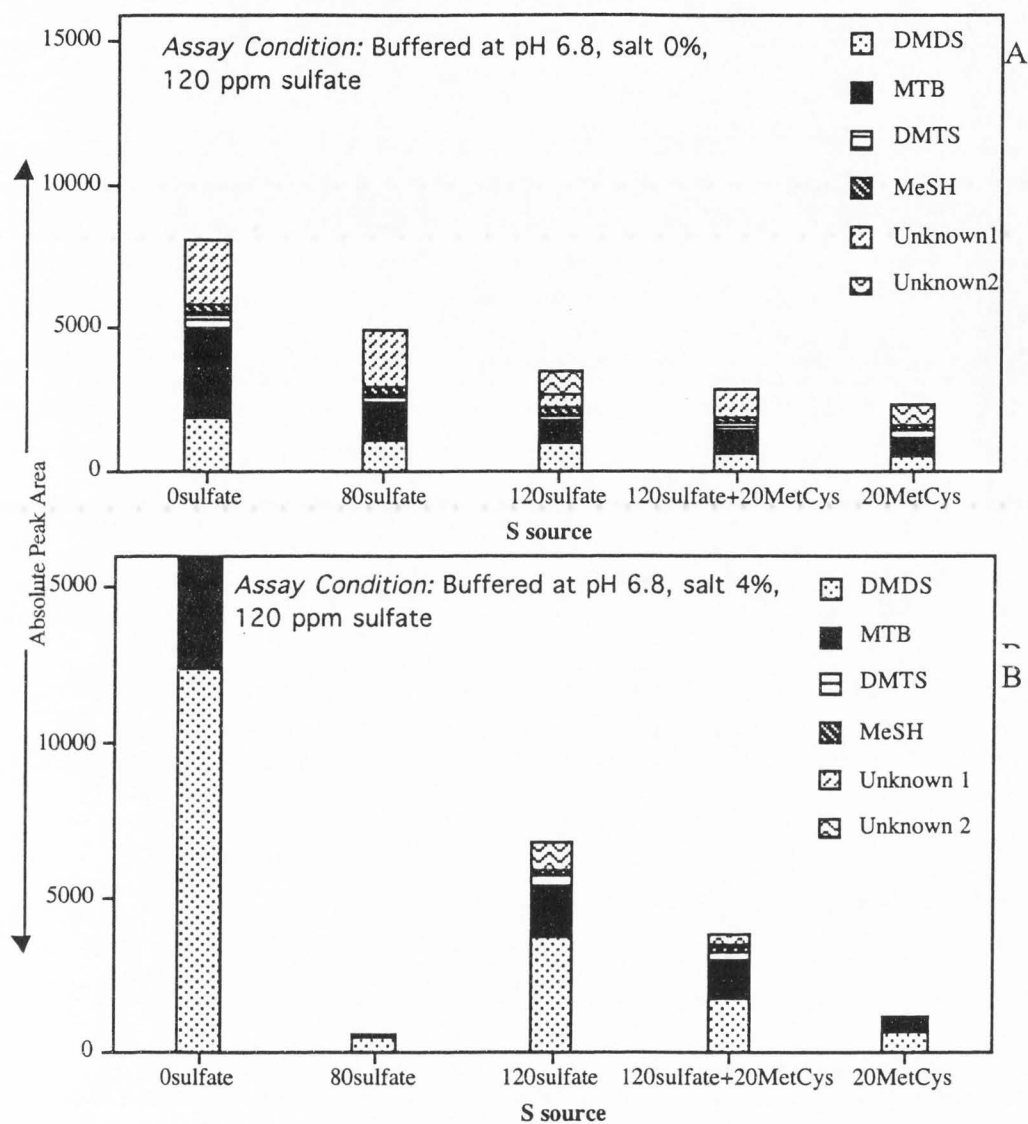


Figure 8. VSC composition in the headspace of *Lactococcus lactis* ML3 after assay with sulfate (pH 6.8) as analyzed by gas chromatography with cryo-focusing.

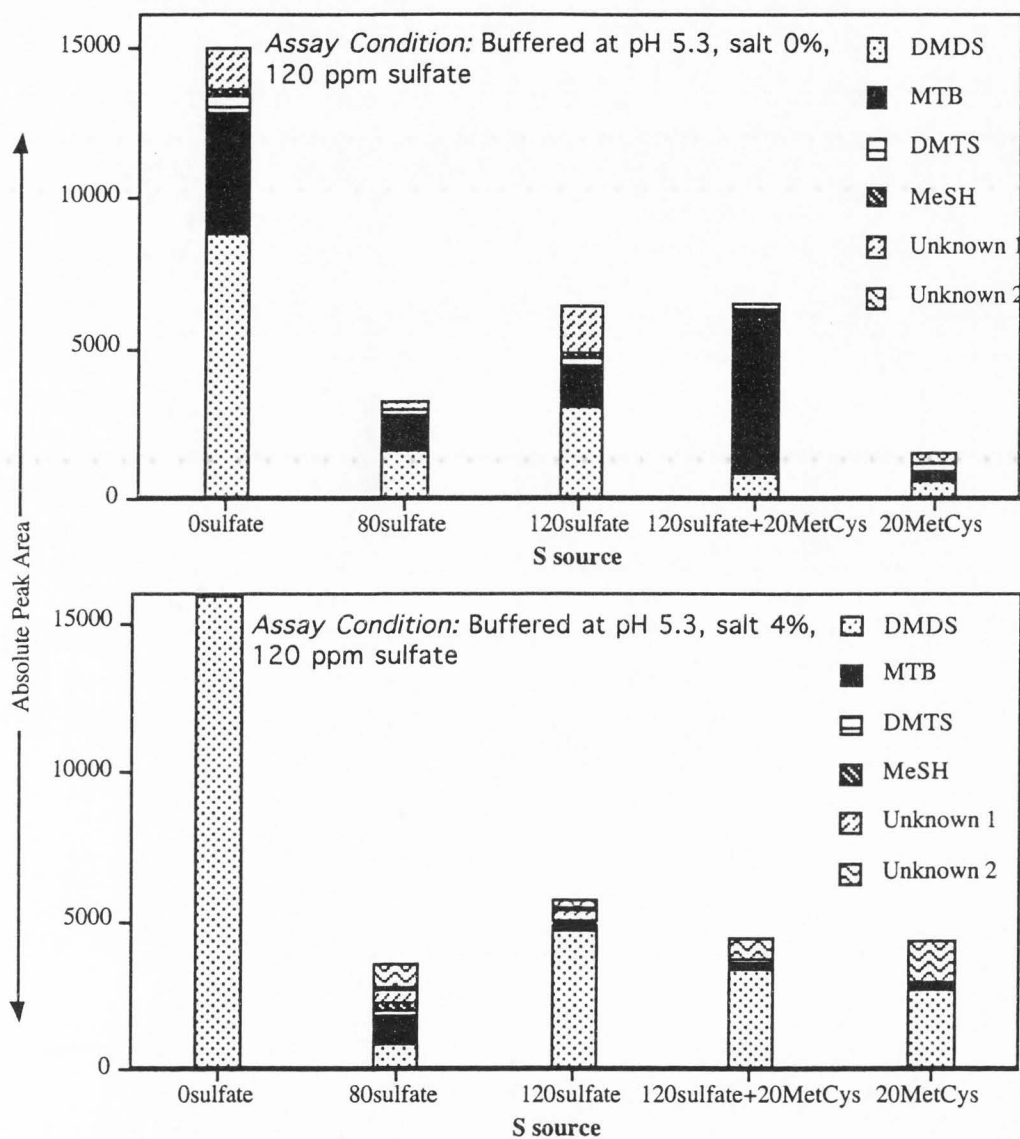


Figure 9. VSC composition of the headspace *Lactococcus lactis* ML3 after assay with sulfate (pH 5.3) as analyzed by gas chromatography with cryo-focusing.

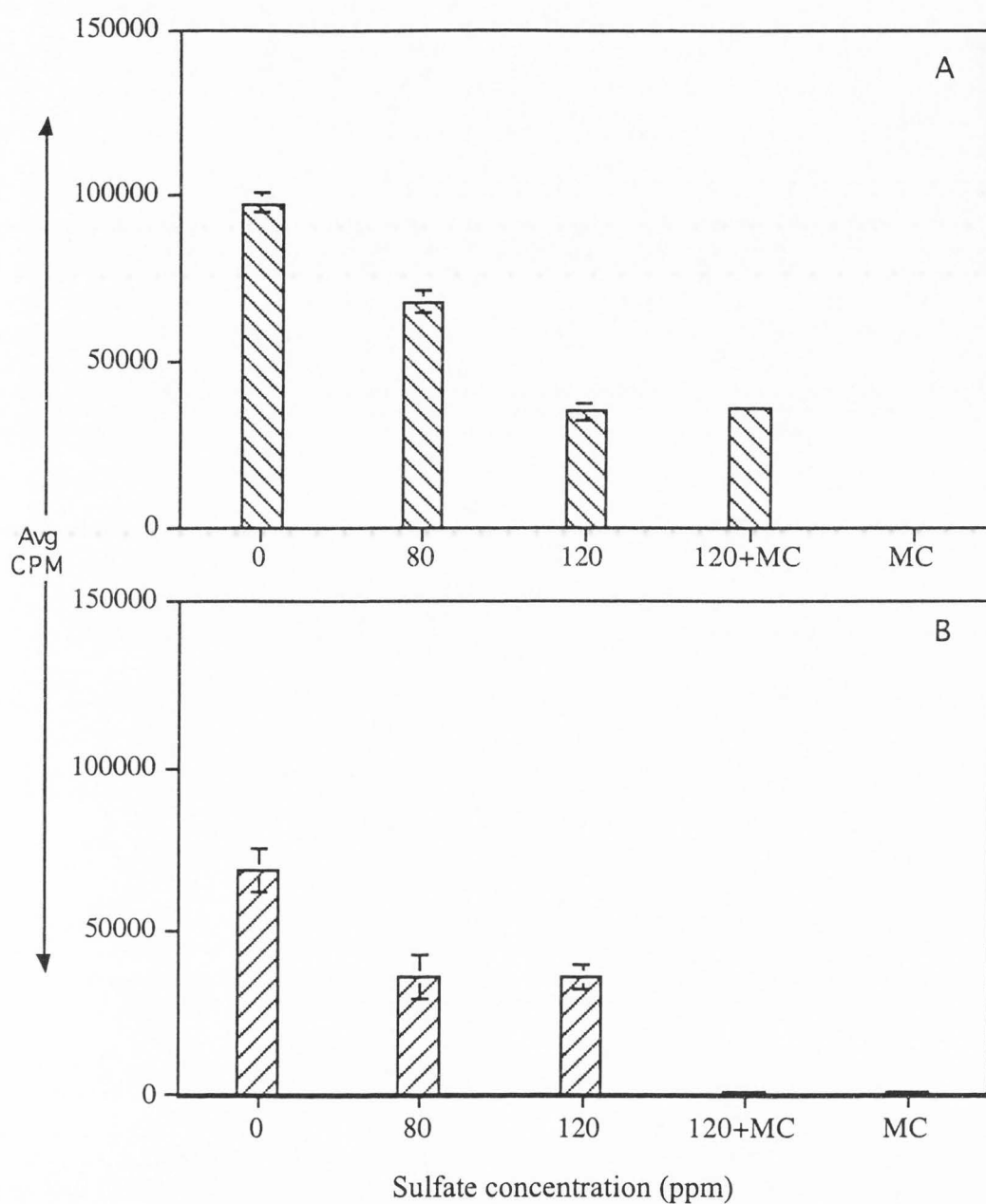


Figure 10. Scintillation count (counts per minute) of a 15 ml sample of headspace of *L. lactis* IL1403 assayed at pH 5.3 (panel A) and pH 6.8 (panel B) for 2 hours with sulfate at different levels as the only source of sulfur during growth and 120 ppm sulfate as the only source of sulfur in assay buffers. (MC = methionine-cysteine in 20 ppm each).

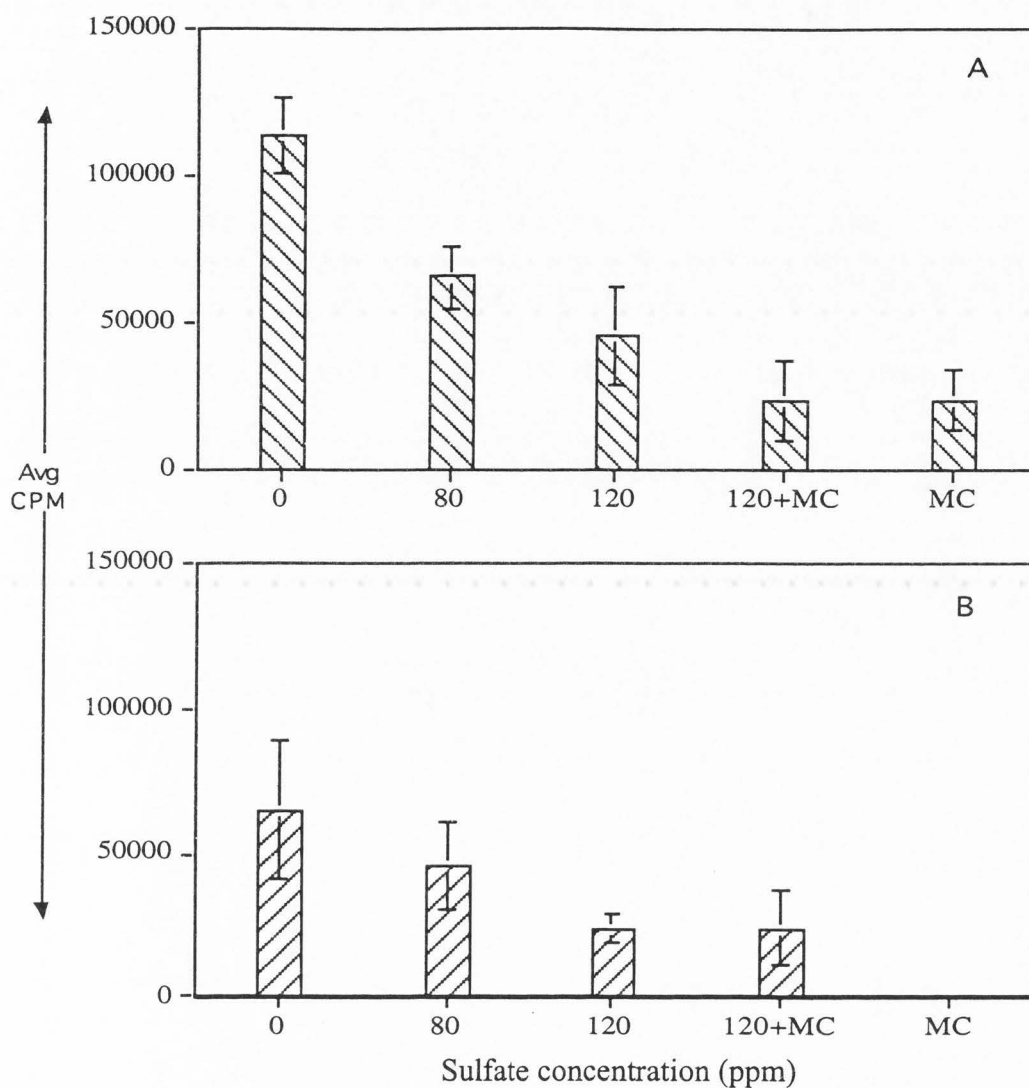


Figure 11. Scintillation count per minute of the 15ml sample of headspace of ML3 assayed in pH 5.3 (panel A) and pH 6.8 (panel B) for 2 hours with sulfate at different levels as the only source of sulfur during growth and 120 ppm sulfate as the only sulfur source in assay buffer. (MC = Methionine-Cysteine in 20 ppm each).

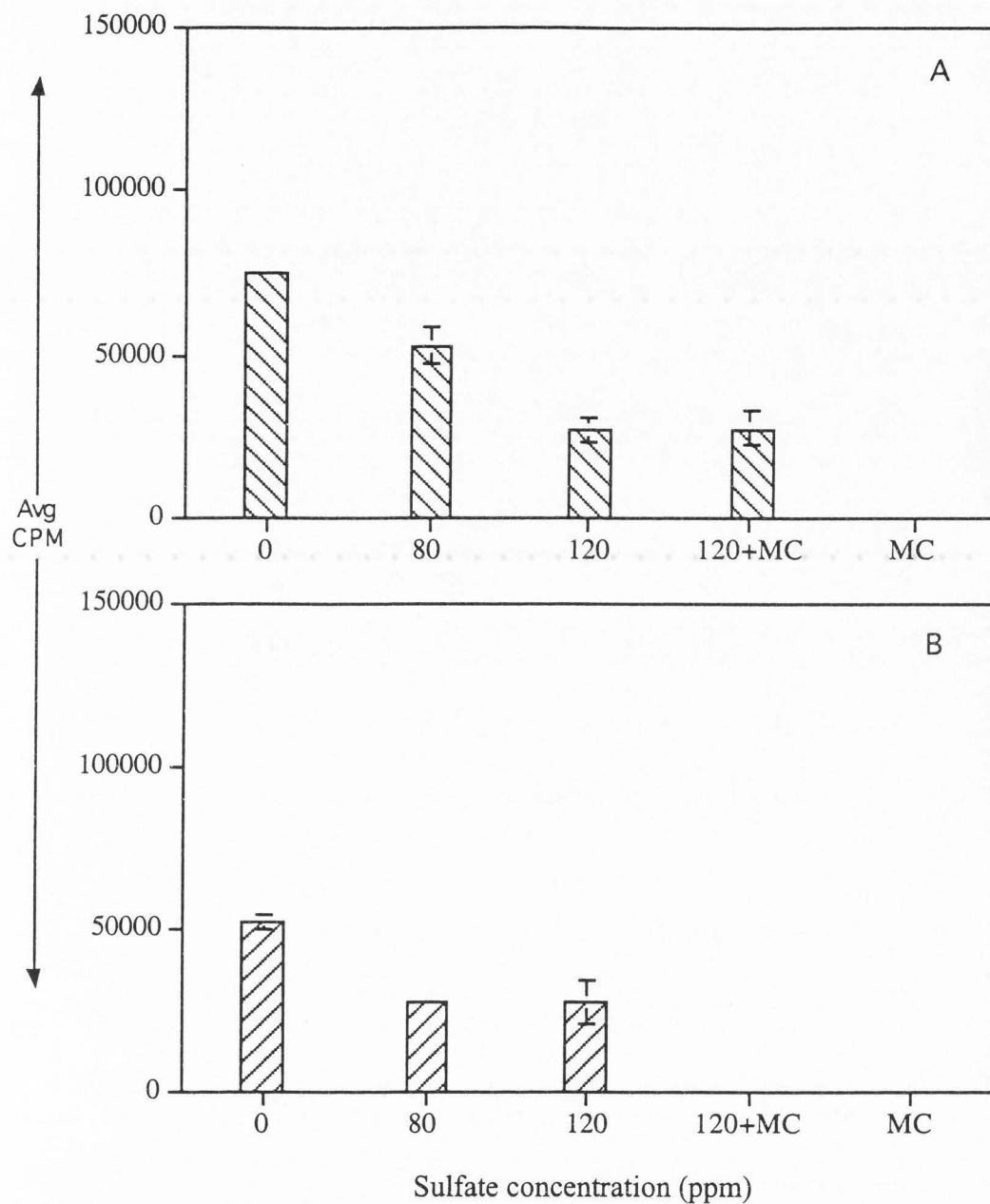


Figure 12. Scintillation count per minute of the 15ml sample of headspace of SK11 assayed in pH 5.3 (panel A) and pH 6.8 (panel B) for 2 hours with sulfate at different levels as the only source of sulfur during growth and 120 ppm sulfate as the only source of sulfur in assay buffers. (MC = methionine-cysteine in 20 ppm each).

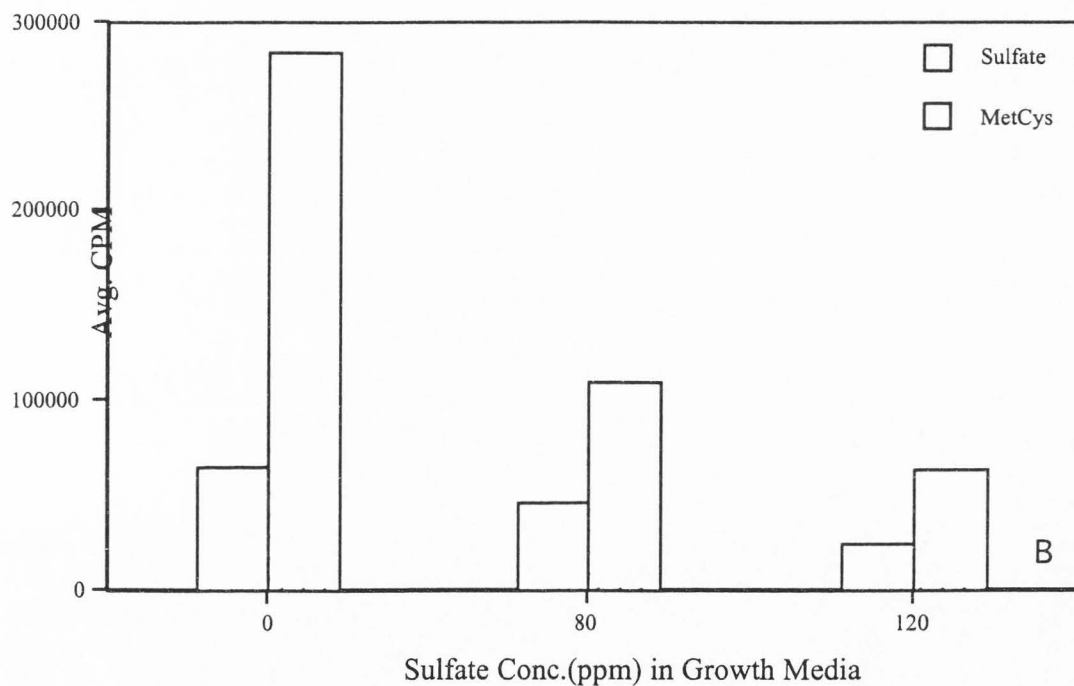
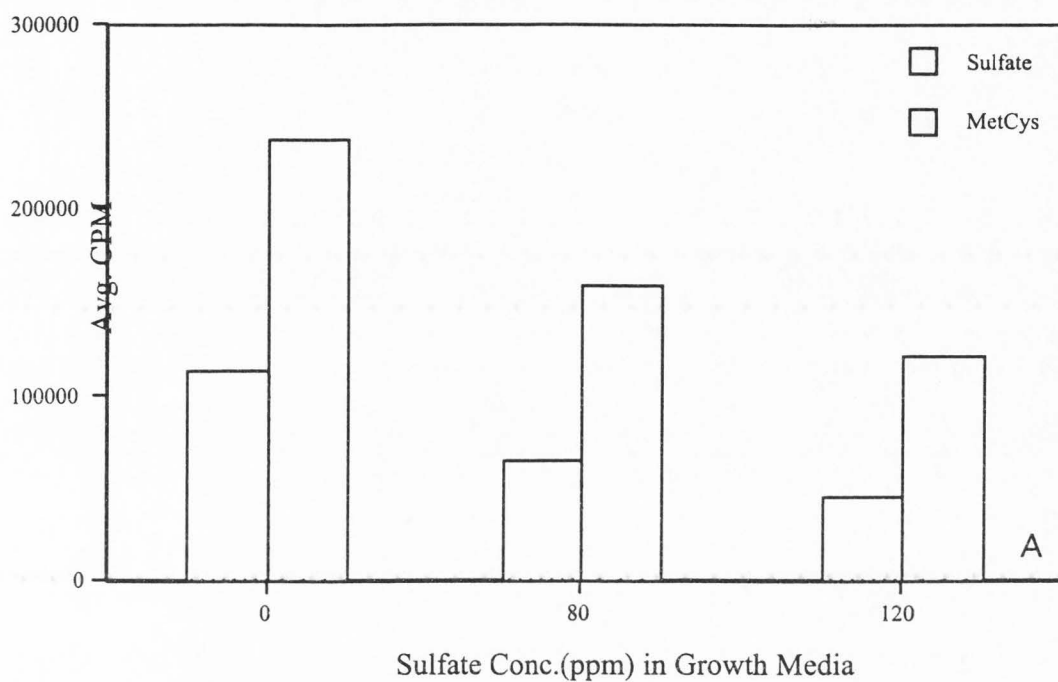


Figure 13. Scintillation count per minute of the 15ml sample of head space of ML3 assayed in pH 5.3 (panel A) and pH 6.8 (panel B) for 2 hours with sulfate at different levels as the only source of sulfur during growth and 120 ppm sulfate and 20 ppm each of methionine and cysteine as sulfur sources in assay buffers.

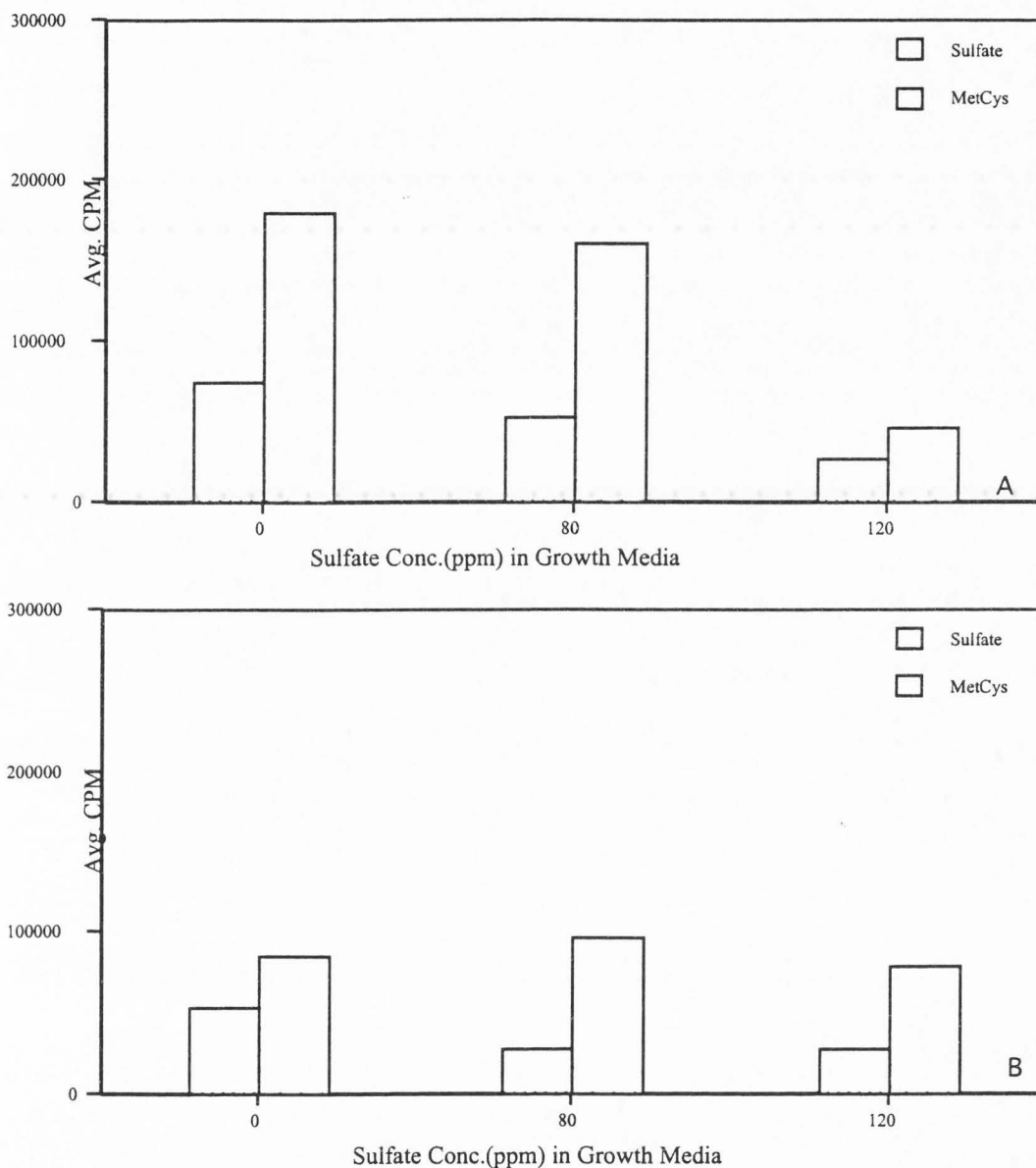


Figure 14. Scintillation count per minute of the 15ml sample of head space of SK11 assayed in pH 5.3 (panel A) and pH 6.8 (panel B) for 2 hours with sulfate at different levels as the only source of sulfur during growth and 120 ppm sulfate and 20 ppm each of methionine and cysteine as sulfur sources in assay buffers.

CHAPTER IV

SUMMARY

The work presented in this thesis was motivated by the lack of detailed understanding of sulfur metabolism in the bacteria associated with cheese, especially Cheddar cheese. VSCs are the only set of documented compounds that directly lead to better quality and flavored cheese. A limited number of enzyme systems have been associated with Met metabolism in lactococci. The only enzymes directly found that produce increased VSCs are those used to interconvert Met and Cys (β -, γ - lyases). These enzymes are transcriptionally down regulated with increasing Met and not active in cheese conditions. Conversely, the methionine concentration increases during Cheddar cheese ripening. These seemingly disparate facts lead us to suspect that an inorganic source of the sulfur atom may have a role in VSC production. This lead to the hypothesis and objectives listed below.

Hypothesis

Lactococci fix sulfur from inorganic sulfate to produce volatile sulfur compounds.

Objectives

1. Determine availability of inorganic sulfate in milk.
2. Determine the effect of environmental pH and cellular growth phase on sulfate transport in lactococci.

3. Determine assimilation of inorganic sulfate into cellular proteins of lactococci.
4. Determine production of VSC from inorganic sulfate by lactococci.

Each objective provided a critical piece of information that allowed the entire set of data to be used to test the validity of the hypothesis. The hypothesis was proven true – milk contains a small, but measurable, amount of sulfate, lactococci do transport sulfate, and they produce VSCs from the inorganic sulfur atom.

The first objective was needed because no published method was available for the determination of sulfate in milk. A number of spectrophotometric methods were attempted before this work began, but they failed to provide a reasonable method that was free from interfering organic compounds. This objective was successfully completed by designing a new method for the determination of total sulfate milk.

The method was investigated for the source of milk, the fat content and the repeatability. The method also provided a sample preparation procedure to remove fat and produce whey for analysis. Briefly, whey from milk was collected for the addition of barium chloride to whey that produces an insoluble barium sulfate precipitate that was directly proportional to the sulfate content. The sulfate concentration was measured with the turbidity at 600 nm of the insoluble salt. The initial work was done in Tris buffer to demonstrate the proof of concept and avoid confounding factors (protein, fat, sugar) in milk. This provided evidence that the method was acceptable ($r^2 = 0.98$) with a dynamic range of ~10-fold.

The sulfate concentration in milk was determined by comparison with standard curve of the sulfate concentration in whey from skim and whole milk ($r^2 = 0.98$). This

method was simple and repeatable (0.65% in skim milk and 1.07% in whole milk) that accurately (RSD = 0.12%) determined the total sulfate concentration in milk with a detection limit that ranged from ~6 to 12 ppm depending on the initial sample type (buffer or whey). Using this method, we found skim and whole milk to be 49.2 and 46.7 ppm, respectively. While skim and whole milk from commercial sources were significantly different ($p < 0.0001$), the difference was not considered practically important because they were different by only ~2 ppm. This was also true of the commercial source. Consequently, this assay was used to measure the residual sulfate as an indicator of transport during lactococcal growth in various media in objective 2.

The second objective was completed by determining if three strains of lactococci had the ability to transport sulfate during growth. Prior to the experimentation, we examined the *L. lactis* IL1403 genome and found that this strain contains a putative membrane sulfate transporter (*yafB*). The transport assays provided evidence that IL1403, as well as ML3 and SK11, removed sulfate from the growth medium. The total sulfate transport increased with a decrease in pH, with ML3 transporting significantly ($p < 0.05$) more sulfate than SK11 and IL1403 at 6.3 and below. More sulfate was transported during growth, opposed to stationary phase cells.

Based on fixation in other organisms, we assumed that the sulfate would be fixed into cellular protein and then converted to VSC via a known pathway in lactococci. To determine protein assimilation of sulfate, we used a 2D PAGE method that has been used with *E. coli*. The method was successfully established with *E. coli* K12 and we observed the expected fixation event with radiolabeled protein spots on the autoradiograph. However, we did not observe sulfate assimilation into cellular protein in any lactococcal

strain tested, despite cellular transport. The lack of sulfate assimilation into protein is confusing, but we speculate that it may be due to the slow growth rate of lactococci compared to *E. coli* or an unknown storage form of sulfate used in lactococci.

Alternatively, this observation was somewhat expected because the genes associated fixation are missing in the IL1403 genome. Considering that ~40% of the genome is annotated as unknown function, it is possible that a paralogue to the genes in other organisms will be found that explain this difference. Our assumption of a protein (or amino acid) being used as the precursor to VSC formation is not clear and may be false. However, we moved to verify that inorganic sulfur is the source of VSCs.

Objective 3 was done to verify that inorganic sulfate is a source of the sulfur atom in VSCs. An assay system using sulfate and Met as the sole sulfur source was used after growth in various sulfur sources. Previous studies have demonstrated that VSC production via Met metabolism is repressed with addition of organic sulfur (Met or Cys) to the growth medium. We expanded this question to investigate the use of inorganic sulfate for regulation of VSC production and compared it to Met and Cys.

The total assay protocol for this objective used cells grown in various sulfur sources followed by an assay in increasing levels of sulfate or Met (and combinations of the two). After incubation, the headspace from the assay was collected for sulfur-specific GC analysis. We observed the total VSC amount significantly ($p < 0.05$) decreased with increasing sulfate concentration and with the addition of Met/Cys during growth. The maximum VSC production was observed during sulfur starvation (no added sulfur source during growth) for all strains tested. This observation confirms that inorganic sulfur also regulates VSC production.

The VSC composition of the headspace was also influenced by the sulfate concentration. DMDS and MTB were the most common and abundant compounds in the headspace of the assay mixture in all the strains. However, the amount of these compounds changed in response to the sulfur concentration and source during growth. These observations confirm that the sulfate concentration regulates the VSC amount and composition; however, it does not directly confirm that inorganic sulfur was used to produce the VSCs in the headspace.

To prove that the inorganic sulfur atom was the source of sulfur for the VSC pool in the headspace, we used the same experimental conditions as the GC experiment, except the sulfate or Met in the assay mixture was radiolabeled and the VSCs in the headspace were measured with scintillation counting. This method collected 15 ml of headspace gas from the assay and injected it into a sealed scintillation vial containing the scintillation cocktail mixture. If the headspace contained radioactivity, the fluor would be excited and it would be detectable by scintillation. Growth of each strain in increasing concentrations of sulfate decreased the radiolabeled VSC content (see Figures 10-12). Only ML3 produced any VSCs with the addition of 20 ppm of methionine/cysteine to the growth medium, demonstrating the regulatory capability of organic sulfur as previously observed. Assay at a lower pH (5.3) increased the VSC production, confirming our previous GC analysis (see Figure 9).

An additional experiment was done to further explore the influence of sulfate on the ability to regulate organic sulfur metabolism to VSCs by growing the cells in increasing sulfate and then assaying the VSC composition with radiolabeled Met/Cys. As expected from our previous data, during sulfur starvation, sulfate was also the source of

the sulfur atom in the VSCs during assay. These experiments demonstrated that sulfur starvation during growth lead to radiolabeled Met/Cys being used as a source of sulfur for VSC in the assay headspace – irrespective of the assay pH. Increasing the sulfate content in the medium decreased VSC production from Met/Cys as well, further confirming that inorganic sulfur regulates VSC production from organic sources as well as regulating VSC production from inorganic sources.

This study successfully completed all the objectives by developing a sulfate assay for milk, demonstrating transport of sulfate in lactococci, and proving that lactococci can fix inorganic sulfur into VSCs via an unknown mechanism. These observations confirm that lactococci fix inorganic sulfur into VSCs; thereby, proving the hypothesis.

To prove the mechanism of the conversion and the regulatory web between inorganic and organic sulfur additional experimentation will need to be done. This may proceed via transcription studies using a whole genome array of IL1403 or the other strains to determine what genes are involved, with particular interest in the unknown genes. Additional studies that incorporate the use of NMR analysis will determine the metabolic intermediates that contain the sulfur atom. Combining these systems will provide a complete picture of the genetic and metabolic metabolism of the sulfur atom in lactococci.

APPENDICES

Appendix 1

Volatile Sulfur Compound Production

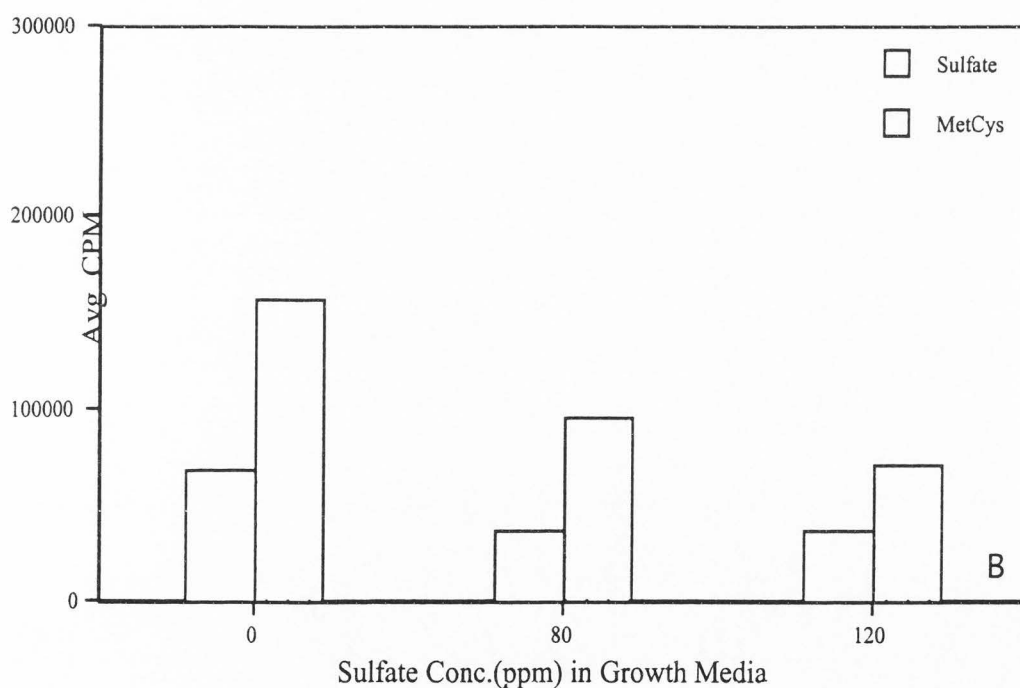
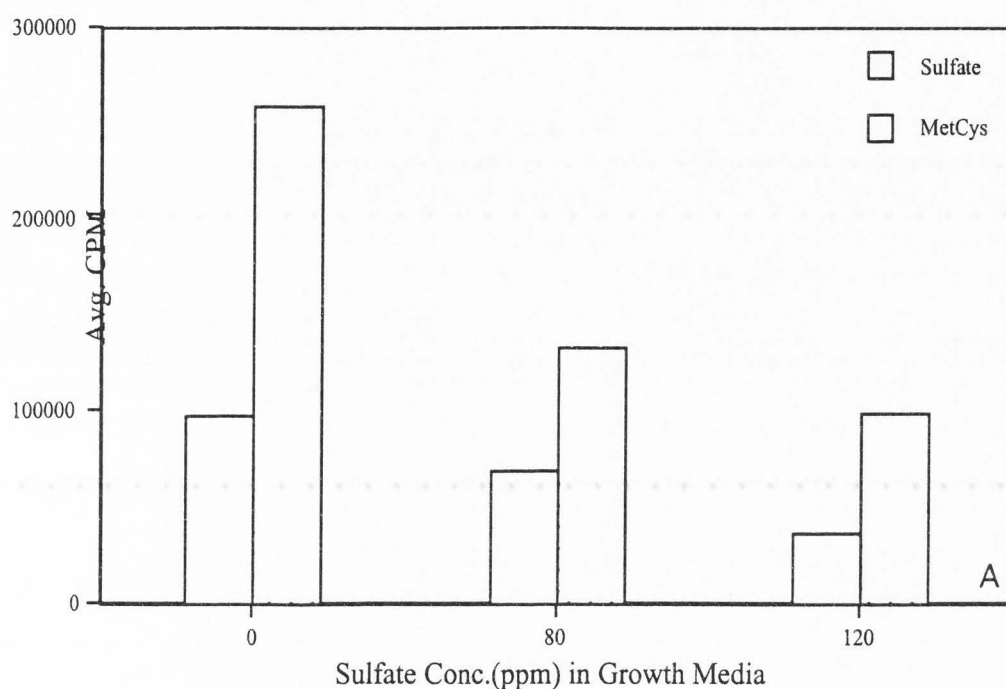


Figure 15. Scintillation count per minute of the 15ml sample of head space of IL1403 assayed in pH 5.3 (panel A) and pH 6.8 (panel B) for 2 hours with sulfate at different levels as the only source of sulfur during growth and 120 ppm sulfate and 20 ppm each of methionine and cysteine as sulfur sources in assay buffers.

APPENDIX 2

Autoradiograms of 2D gels

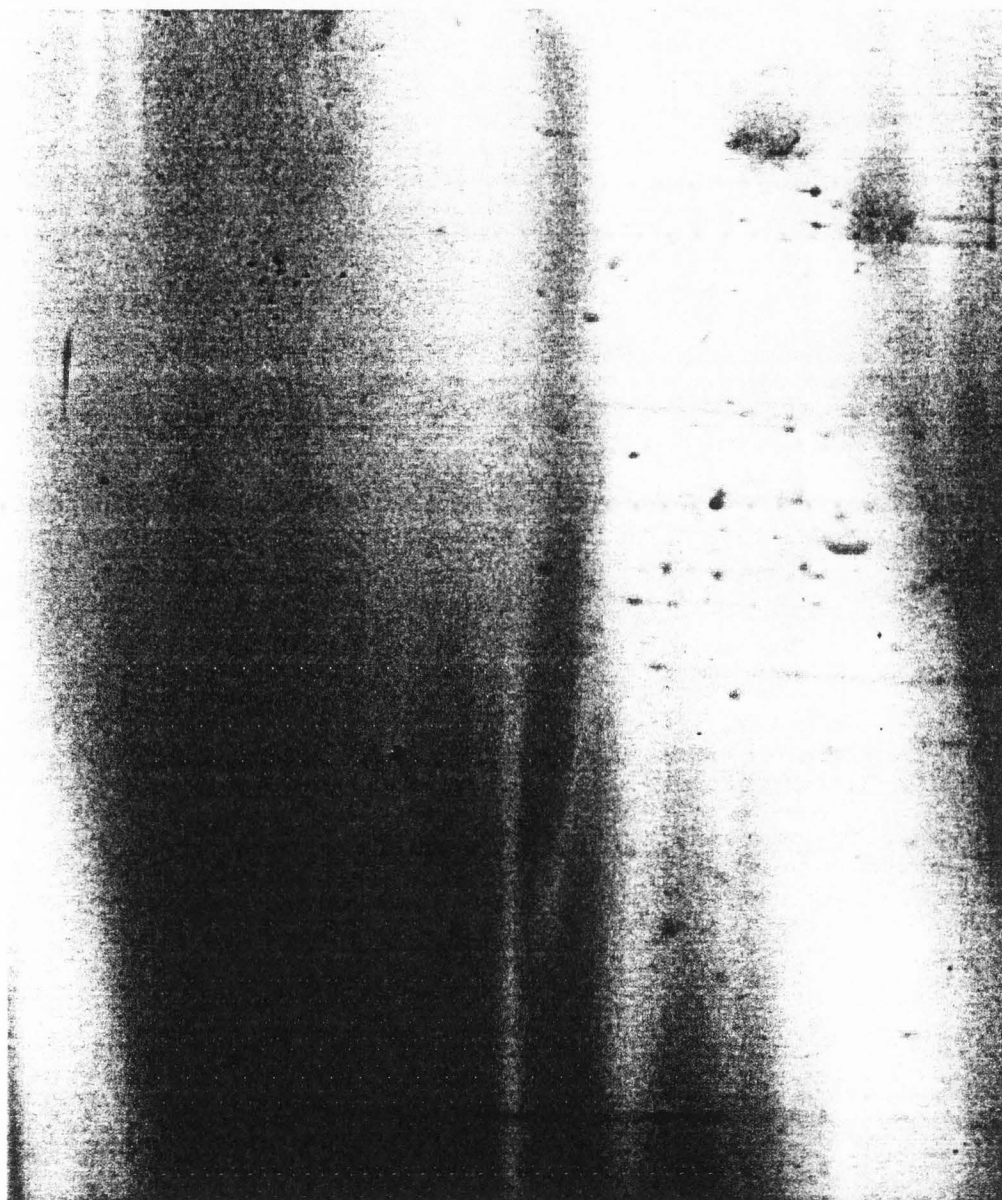


Figure 16. X-OMAT AR film showing radioactive protein spots upon exposure by total protein 2D PAGE of *E.coli* cell lysate after growing it in CDM with ^{35}S -sulfate as the sole source of sulfur. Note: the contrast and brightness of this image was modified to enhance the visualization of the protein spots.



Figure 17. X-OMAT AR film showing radioactive protein spots upon exposure by total protein 2D PAGE of *Lactococcus lactis* ML3 cell lysate after growing it in CDM with ^{35}S -methionine-cysteine as the sole source of sulfur. Note: the contrast and brightness of this image was modified to enhance the visualization of the protein spots.

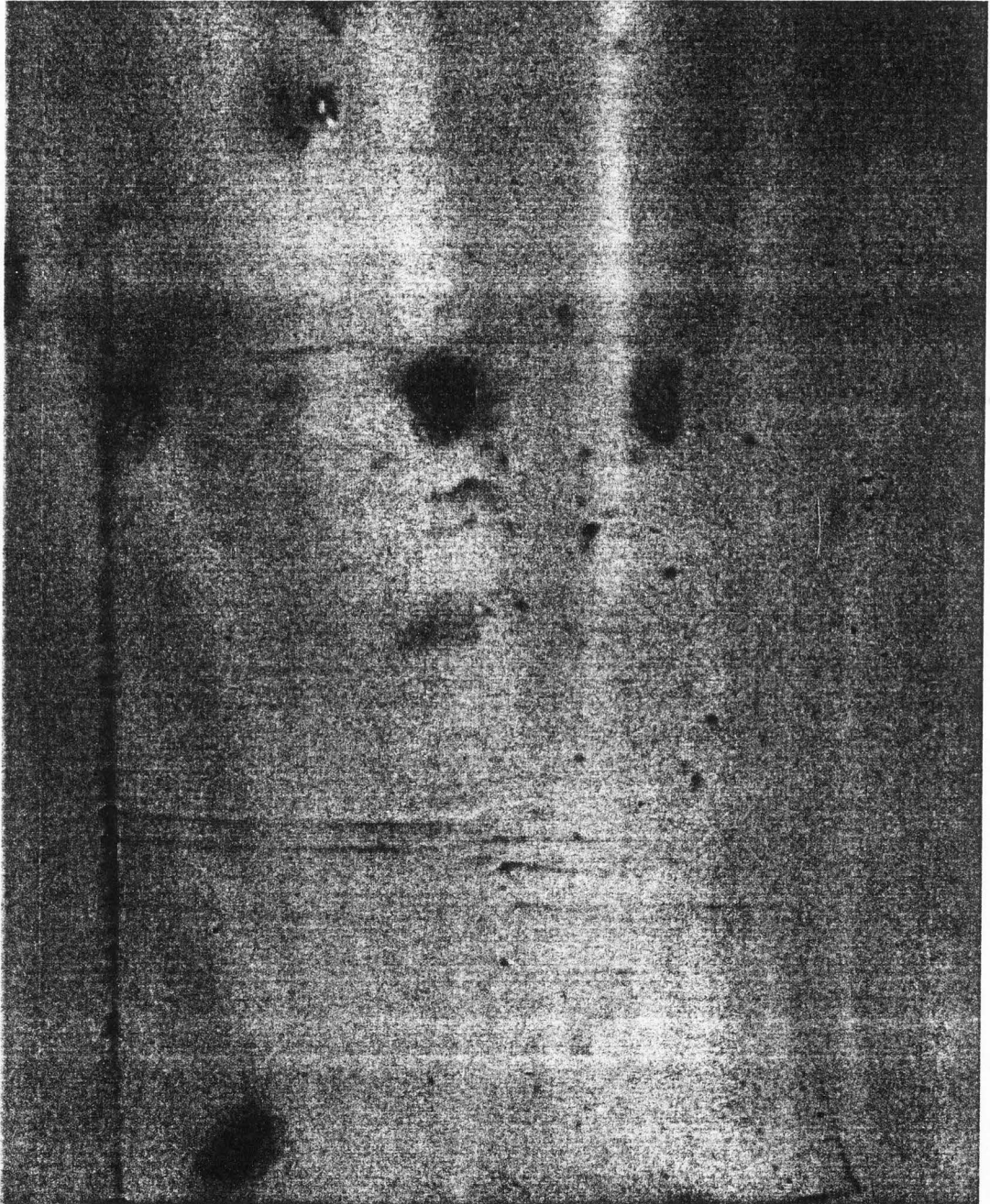


Figure 18. X-OMAT AR film showing radioactive protein spots upon exposure by total protein 2D PAGE of *Lactococcus cremoris* SK11 cell lysate after growing it in CDM with ^{35}S -methionine-cysteine as the sole source of sulfur. Note: the contrast and brightness of this image was modified to enhance the visualization of the protein spots.



Figure 19. X-OMAT AR film showing radioactive protein spots upon exposure by total protein 2D PAGE of *Lactococcus lactis* IL1403 cell lysate after growing it in CDM with ^{35}S -methionine-cysteine as the sole source of sulfur. Note: the contrast and brightness of this image was modified to enhance the visualization of the protein spots.

TABLE 4. Composition of Chemically Defined Medium (devoid of sulfur) used in this study.

Constituent	Amount
L-Ala	100 mg/L
L-Arg	50 mg/L
L-Asn	100 mg/L
Gly	100 mg/L
L-Gln	100 mg/L
L-His	50 mg/L
L-Ile	20 mg/L
L-Leu	20 mg/L
L-Lys	100 mg/L
L-Pro	200 mg/L
DL-Ser	100 mg/L
DL-Thr	100 mg/L
DL-Val	100 mg/L
L-Aspa	100 mg/L
L-Glua	400 mg/L
L-Trpa	25 mg/L
L-Pheb	20 mg/L
L-Tyrb	50 mg/L
Na-acetate	5000 mg/L
Na-citrate	2000 mg/L
K-phosphate (monobasic)	1000 mg/L
K-phosphate (dibasic)	1000 mg/L
NaCl	200 mg/L
CaCl ₂	200 mg/L
MgCl ₂	200 mg/L
MnCl ₂	50 mg/L
Pyridoxal	5 mg/L
Pyridoxamine	5 mg/L
Adenine	25 mg/L
Guanine	25 mg/L
Uracil	25 mg/L
Xanthine	25 mg/L
Tween 80	1 mL
Tween 20	1 mL
Glycerol	1 mL
Mevalonic acid lactone	0.1 mL
Vitamins	20 mL
Trace Elements	2.5 mL